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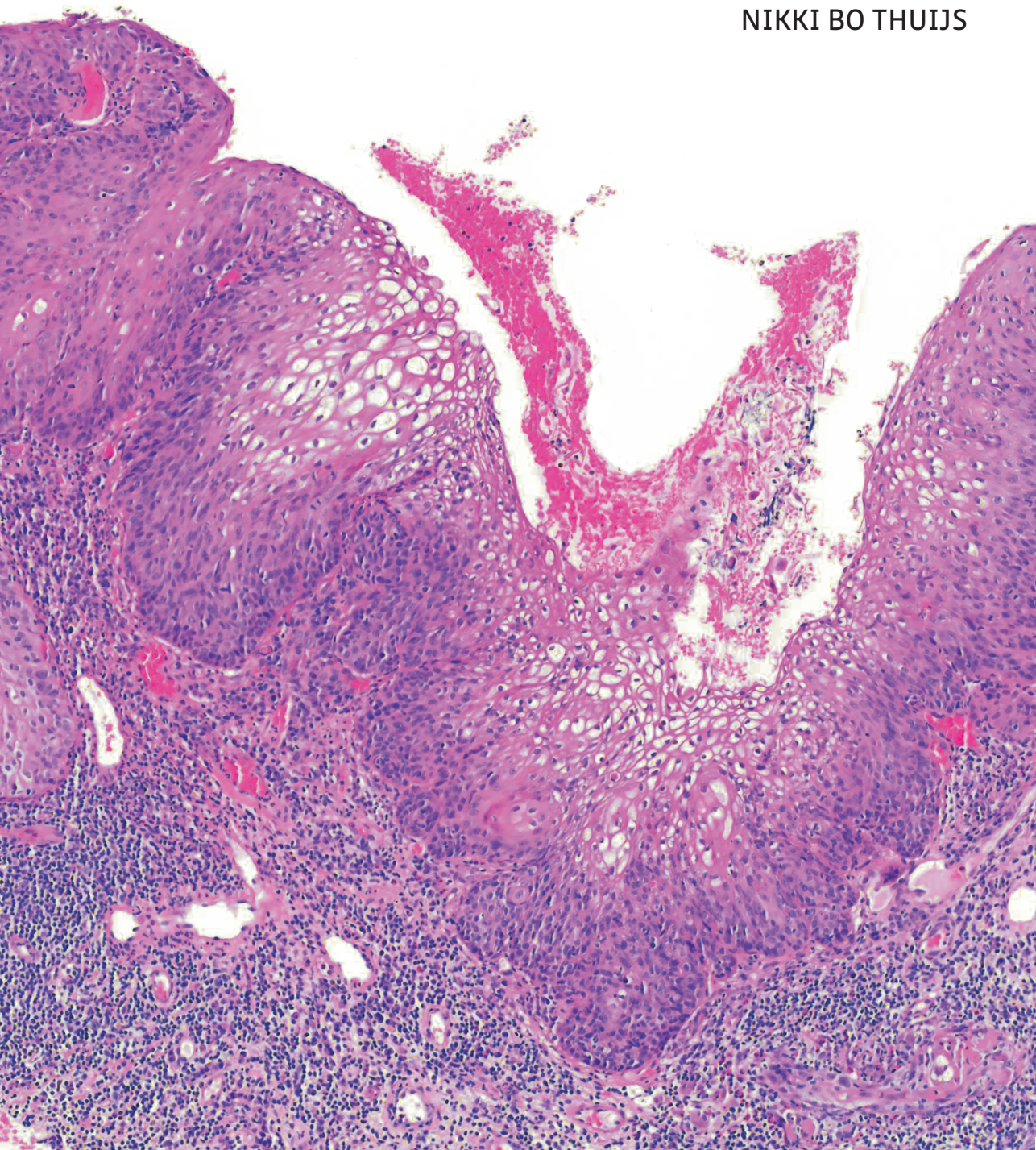

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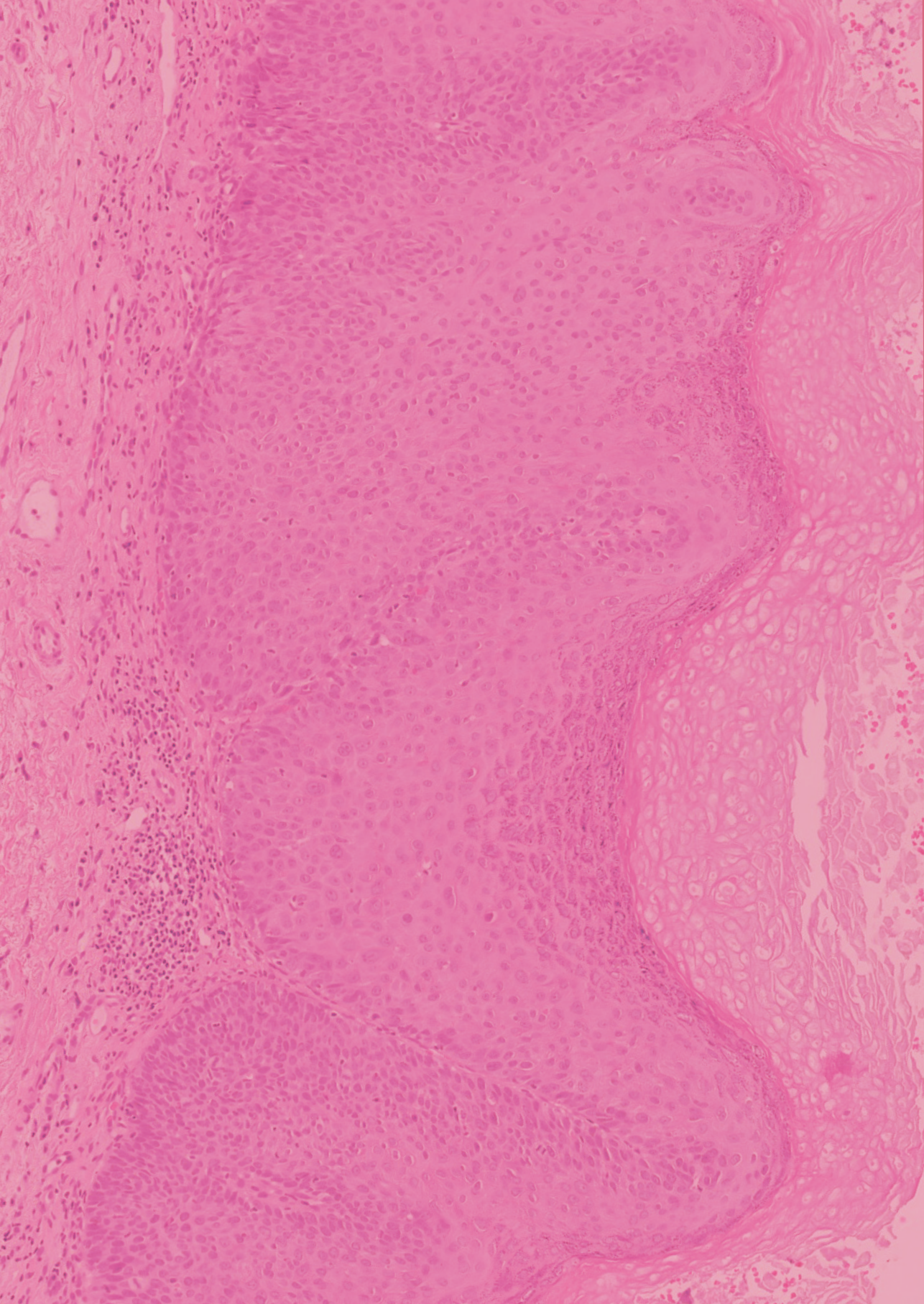
Voor mijn dierbaren

met jullie is alles leuker

TABLE OF CONTENTS



CHAPTER 1

General introduction and thesis outline

Introduction

Vulvar squamous cell carcinoma is the most common cancer of the vulva with 460 new cases in the Netherlands in 2023, corresponding to an annual incidence of 5.1 per 100,000 women.[1] The worldwide incidence of vulvar cancer increased in the last decades, especially in younger patients, although vulvar cancer usually presents around the age of 70 years.[2-4] The precursor lesion of vulvar cancer is high-grade vulvar intraepithelial neoplasia (VIN). The reported incidence of VIN in the United States is 3.9 per 100,000 women.[5] Over the last 30 years, the incidence of VIN also increased.[4, 6]

VIN is currently categorized in high-grade squamous intra-epithelial lesion (HSIL) and HPV-independent VIN. HPV-independent VIN is often referred to as differentiated VIN (dVIN). These two subtypes are separate disease entities with different causative agents, histopathology, clinical presentation and prognosis (Figure 1).[7] HSIL is caused by high-risk HPV (hr-HPV) infection, whereas HPV-independent VIN is associated with vulvar dermatoses like lichen sclerosus (LS), independent of HPV. HSIL is the most common type of VIN, accounting for approximately 90% of cases, but accounts for only 25% of all vulvar cancers. HPV-independent VIN represents 10% of all VIN and gives rise to 75% of vulvar cancers.[8] HPV-independent VIN is often diagnosed in the context of vulvar cancer, e.g. at time of vulvar cancer diagnosis or during follow-up of vulvar cancer.

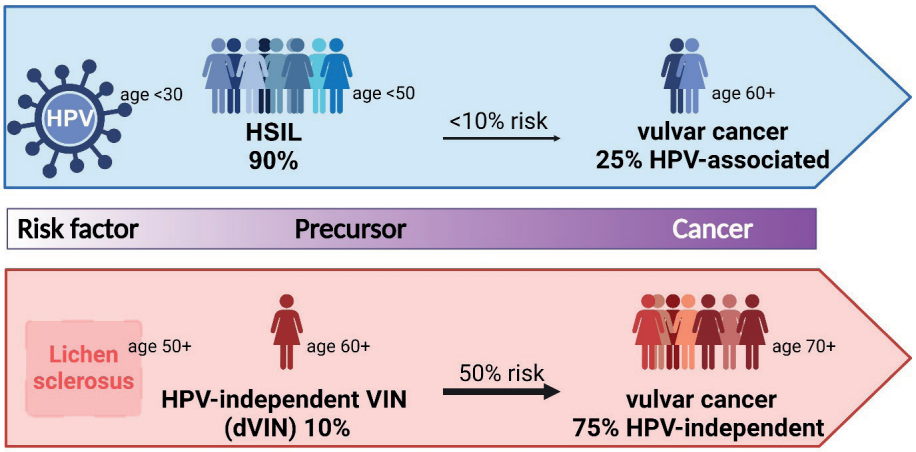


Figure 1. Pathways to vulvar cancer. Created with Biorender.com.

Structures belonging to the vulva are the mons pubis, anterior and posterior commissure, labia majora and minora, clitoris, external urethral orifice, vestibule of the vagina, perineum, and peri-anal region (Figure 2). The labia majora fuse anteriorly

into the mons pubis and posteriorly into the perineum. The vestibule is the area between the labia minora containing the urethral opening and introitus (vaginal opening). The vulva is covered with stratified squamous epithelium. The hear-bearing skin of the labia majora consists of the epidermis, dermis and subcutis. The mucosal surface covering the central part of the vulva consists of non-keratinizing squamous epithelium with underlying stroma (Figure 3).

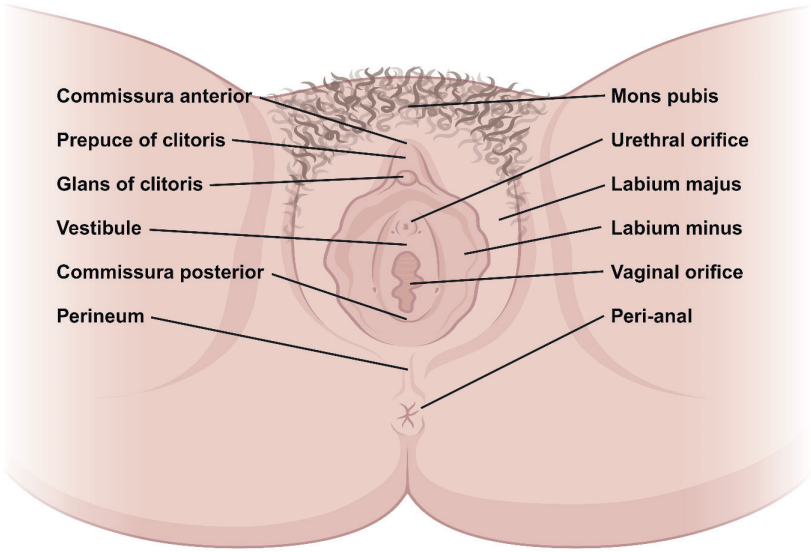


Figure 2. The vulvar anatomy. Created with Biorender.com.

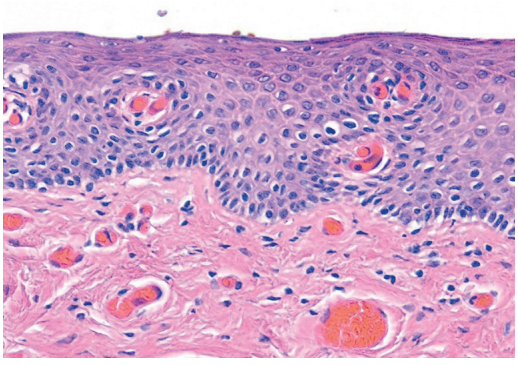


Figure 3. Histology of healthy vulvar tissue. The epithelial cells (keratinocytes) mature as they differentiate upwards. The basal cell layer consists of regularly spaced, small cells with little cytoplasm. The differentiated cells at the top of the epithelium have ample cytoplasm and a flattened nucleus.

Classification of VIN

Since VIN was first recognized a century ago, multiple terminologies and classifications have been used (Table 1). Bowen's disease in general (on the shaft of the penis, buttocks, and thighs) was first described by dermatologist J. Bowen in 1912.[9] In 1922, Hudelo et al. recognized the histological features of Bowen's disease of the vulva and named the disease 'erythroplasiform dyskeratosis of the vulvar mucosa'.[10] Almost 40 years later, two types of VIN were described by Abell and Gosling: intraepithelial carcinoma of i) Bowen's type, and ii) simplex type. In 1976, the International Society for the Study of Vulvovaginal Disease (ISSVD) introduced 'squamous cell carcinoma in situ' for 'Bowenoid' lesions and 'hyperplastic dystrophy with mild, moderate, or severe atypia' for 'simplex' lesions, because multiple confusing terms were used at that time. The term 'differentiated VIN' (dVIN) was introduced several years later, to emphasize the differentiated histomorphology of the 'simplex' VIN type.[11]

Nowadays, the WHO 2020 classification of female genital tumors subdivides VIN based on etiology into HPV-associated low-grade squamous intraepithelial lesion (LSIL) or HSIL, and HPV-independent VIN.[12] HPV-independent VIN also includes vulvar acanthosis with altered differentiation (VAAD) and differentiated exophytic vulvar intraepithelial lesion (DEVIL).[12-14] There is still a lot of debate on the nomenclature and classification of the different histomorphological subtypes of HPV-independent VIN. The ISSVD recognizes besides dVIN, the term vulvar aberrant maturation (VAM) for DEVIL, VAAD and other related p53 wild-type lesions.[15] Other authors have recently grouped verruciform lichen simplex chronicus, VAAD and DEVIL under the term verruciform acanthotic VIN (vaVIN).[16] Although it is important to recognize the morphological spectrum of VIN, there is a need for an objective classification that reflects both biological and clinically relevant features.

Vulvar HSIL

Vulvar HSIL most commonly affects patients aged 30 to 50 years, although also older patients present with the disease.[4] HSIL is often symptomatic, with long-lasting pruritus and pain, but the clinical presentation is variable and lesions can be discovered only after a long time (Figure 4A). Twenty-five to 66% of patients suffer from multifocal (multiple vulvar HSIL) or multicentric (intraepithelial lesions at other anogenital sites, such as the cervix (CIN), vagina (VaIN), and/or anus (AIN)) disease.[17, 18] HSIL is caused by hr-HPV infection and in 70 to 90% of vulvar HSIL, hr-HPV is detected.[8, 19] Risk factors for HSIL are smoking and immunosuppression, including HIV infection.[20-22] HSIL recurs in 20 to 60% and recurrence is more frequently observed in immunocompromised patients and patients with multifocal or multicentric disease.[20, 23]

Table 1. Nomenclature used for VIN of the vulva in the last century.

Year	Author	Terminology
1922	Hudelo et al.	Bowen's disease of the vulva: 'erythroplasiform dyskeratosis of the vulvar mucosa '
1958	Woodruff and Hildebrandt	Carcinoma in situ (CIS)
1961	Abell and Gosling	Intraepithelial carcinoma of i) Bowen's type, ii) simplex type
1967	R.M. Richart	Vulvar intraepithelial neoplasia (VIN)
1976	ISSVD	Squamous cell carcinoma in situ and hyperplastic dystrophy with (mild, moderate, severe) atypia
1977	W.R. Hart	Differentiated VIN (dVIN)
1979	Wade, Kopf and Ackerman	Bowenoid papulosis
1986	ISSVD	VIN1-3, dVIN
1989	WHO	VIN1-3
1994	WHO	Squamous intraepithelial lesion (SIL)
2003	WHO	VIN1-3, dVIN
2004	ISSVD	Condyloma (HPV-effect), VIN usual type, dVIN
2012	LAST	LSIL, HSIL
2014	WHO	LSIL, HSIL, dVIN
2015	ISSVD	LSIL (including flat condyloma / HPV effect), HSIL, dVIN
2020	WHO	HPV-associated SIL (LSIL, HSIL), HPV-independent VIN (dVIN, VAAD, DEVIL)
2021	ISSVD	Addition: vulvar aberrant maturation (VAM) for DEVIL, VAAD and other related p53 wild-type lesions
2022	Parra-Herran, et al.	Addition: verruciform acanthotic VIN (VaVIN) for lesions as verruciform lichen simplex chronicus, VAAD and DEVIL

Abbreviations: DEVIL, differentiated exophytic vulvar intraepithelial lesion; dVIN, differentiated vulvar intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; VAAD, vulvar acanthosis with altered differentiation; VAM, vulvar aberrant maturation; VaVIN, verruciform acanthotic VIN; VIN, vulvar intraepithelial neoplasia.

At the histology level, HSIL is characterized by acanthosis, papillomatosis, hyperkeratosis and/or parakeratosis (Figure 4B). The epithelial maturation is disturbed, with presence of dysplastic cells and mitotic figures up to the upper layers of the epithelium. Cells with little cytoplasm and enlarged, hyperchromatic nuclei, including increased mitoses, and viral or 'koilocytic-like' changes, can be present. Like in all HPV-associated high-grade precursor lesions, $p16^{INK4a}$ shows diffuse, block-like (positive) staining (Figure 4C).

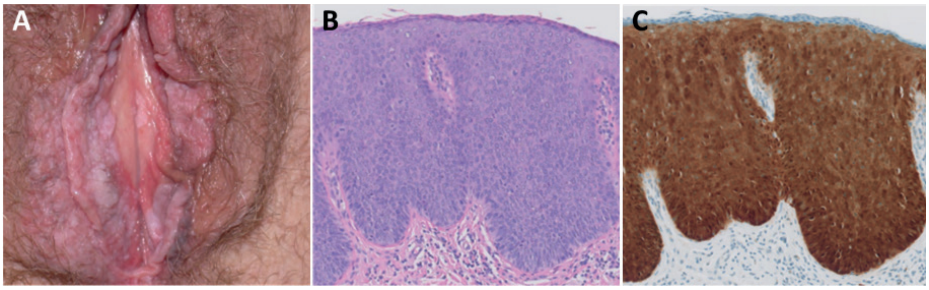


Figure 4. Vulvar high-grade squamous intraepithelial lesion (HSIL).

(**A**) Representative macroscopic image of HSIL consisting of multifocal, white, hyperkeratotic plaques on the labia minora and majora and the introitus, on a background of red colorations.

(**B**) Hematoxylin and Eosin (H&E) staining of HSIL shows acanthosis, disturbed epithelial maturation, i.e. dysplasia, across all layers, with increased number of mitoses and subtle parakeratosis.

(**C**) Block-positive $p16^{INK4a}$ immunohistochemical staining across the full epithelial thickness.

Around 1960, all patients with VIN were surgically treated with full or deep vulvectomy.[24] In subsequent decades, the treatment of VIN became less aggressive and increasingly individualized, depending on patient and lesional characteristics, and through shared decision-making. The optimal management of HSIL is challenging as there is no universal management or gold standard. Nowadays, the aim is preservation of normal anatomy, symptom relief, and high quality of life.[25, 26] Treatment of HSIL often balances between reducing the patients' symptoms and estimating the risk of progression to cancer. Treatment options include topical application with imiquimod, local excision, and laser evaporisation.[25, 27, 28] Large surgical excision can lead to disfigurement and impaired sexual function.[29] Especially in case of small, unifocal HSIL, imiquimod be considered as first-line treatment.[30] Imiquimod is an immune response-modifying drug with antiviral and antitumor activity, first described in 1985.[31] Imiquimod became available in The Netherlands around the year 2005.[32]

In treated HSIL patients, progression rates range from 2 to 6% after an average follow-up of three years, and progression rates increase with longer follow-up time.[33, 34] Spontaneous regression is rare and particularly seen in young patients with multifocal, small, pigmented lesions, often observed during pregnancy.[20, 23, 35] Current prophylactic HPV vaccines offer protection against HSIL and related cancers, in HPV-negative women.[36, 37]

Clinical needs

Since HSIL has a low cancer risk, only HSIL with high cancer risk should be treated extensively. However, current clinicopathological parameters are insufficient to accurately predict the risk of progression to vulvar cancer in HSIL. As a result, there is overtreatment of the HSIL with low cancer risk. Biomarkers that can accurately discriminate HSIL with a high risk of progression to cancer from HSIL with a low risk are very much needed. Those biomarkers will contribute to more individualized therapy, reduced overtreatment and morbidity, and increased quality of life for HSIL patients.

HPV-independent VIN

HPV-independent VIN is commonly found in the 6th to 8th decades of life, but can occur in younger patients.[38] LS, pruritus and/or pain are commonly present.[39] HPV-independent VIN is often unifocal and is difficult to distinguish from its associated dermatosis, making a timely and accurate diagnosis frequently challenging (**Figure 5A**).[40]

The majority of HPV-independent VIN have differentiated morphology, e.g. showing abrupt premature (deep) individual cell keratinization, often accompanied by basal cytological atypia including hyperchromatic, angulated nuclei with prominent nucleoli, loss of granular cell layer, and parakeratosis (**Figure 5B**).[41, 42] However, HPV-independent VIN has a broad histomorphological spectrum, from very subtle to overt dysplastic.[43] These histomorphological differences can be observed between patients and within biopsies of one patient. As a result, HPV-independent VIN can be a difficult diagnosis, even for experienced gynecological pathologists.[43] The main differential diagnoses of HPV-independent VIN are reactive, non-dysplastic lesions.[44] The pathogenesis of HPV-independent VIN is not completely understood. Chronic inflammation as seen in LS and other inflammatory conditions with associated chronic oxidative stress, caused by dermal sclerosis and thickened basement membrane, is believed to be a predisposing factor for DNA damage and subsequent development of HPV-independent VIN.[45, 46] Only in the last decade a clonal relationship between HPV-independent VIN and HPV-independent vulvar cancer was demonstrated, mainly based upon mutations in tumor suppressor gene *TP53*, observed in approximately 70% of HPV-independent VIN (**Figure 5C**).[38, 47-49]

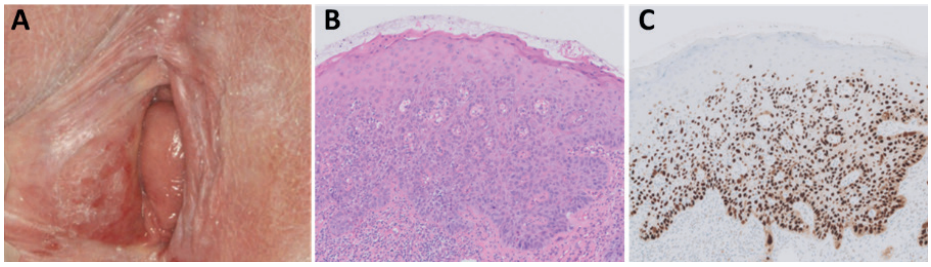


Figure 5. Human papillomavirus-independent vulvar intraepithelial neoplasia.

(**A**) Representative macroscopic image of HPV-independent VIN consisting of multifocal, red colorations and ulcerations on the labia minora and the introitus, on a background of lichen sclerosus (LS). Bilateral fused labia with whitening and thinning of the epithelium, characteristic for LS, are depicted in this macrograph.

(**B**) Hematoxylin and Eosin (H&E) staining of HPV-independent VIN demonstrating acanthosis, dysplasia and increased number of mitoses in the lower epithelial layers, loss of the granular cell layer and subtle parakeratosis. The epithelial maturation is abrupt and present in the lowest parabasal cell layers. The background demonstrates a heavy inflammatory infiltrate.

(**C**) P53 immunohistochemical staining showing a mutant positive pattern in the basal and parabasal epithelial layers, consistent with staining of the dysplastic cells.

For HPV-independent VIN, surgical excision is the preferred treatment option with close follow-up thereafter.[25] Laser and imiquimod should be avoided. The treatment of a patient with HPV-independent VIN should be conducted in a center with special expertise in vulvar pathology.[50] Adequate treatment of concurrent LS with high-potency topical corticosteroids is necessary since therapy compliance decreases the risk of HPV-independent VIN and vulvar cancer.[51, 52]

Cancer risk studies on HPV-independent VIN remain scarce, with few studies having reported cancer risks after treatment, varying between 33% and 86%, within a median time interval of 0.7 to 13 years.[53, 54]

Clinical needs

As the vast majority of vulvar cancers are HPV-independent, early recognition of the HPV-independent precursors is of utmost importance. To date, many patients present with HPV-independent vulvar cancer without a preceding VIN diagnosis, because of poor recognition of HPV-independent VIN, both clinically, histopathologically, and by LS patients themselves.[55, 56] In contrast to the high cancer risk of HPV-independent VIN of 50%, LS has a low cancer risk of 4-7%, while the incidence of LS is significantly higher compared to HPV-independent VIN (respectively 14.9 versus 0.1 per 100,000 women-years).[4, 57, 58] Early detection of HPV-independent VIN is of utmost importance to reduce morbidity from advanced disease and to

improve outcome and quality of life of these patients. Biomarkers that can help to better identify HPV-independent VIN, and to predict the risk of progression to vulvar cancer in these patients, are lacking, but very much needed.

Biomarkers

Human papillomavirus

HPV is a double-stranded deoxyribonucleic acid (DNA) virus, of which more than 200 genotypes have been reported.[59] The lifetime risk to become infected with HPV is larger than 80%.[60] Most HPV infections are cleared by the immune system within two years, but when hr-HPV persists, (pre-)malignant lesions can develop.[61]

The presence of HPV DNA can be used as biomarker in the diagnosis of VIN. Twelve hr-HPV genotypes have been classified as carcinogenic agents in humans by the International Agency for Research on Cancer (IARC), on the basis of epidemiologic and mechanistic evidence: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59.[62] Type 68 has been classified as 'probably' carcinogenic and types 26, 53, 66, 67 and 70, 73, and 82 as 'possibly' carcinogenic.[63] In vulvar HSIL, HPV16 is the most common type (72-77%), followed by HPV33 (8-11%), and HPV18 (3-5%).[64]

Failure of the immune system to control and clear an hr-HPV infection can result in deregulated expression of the viral proteins E6 and E7.[65] Hr-HPV E6 and E7 oncoproteins interact amongst others with p53 and pRb. E6 degrades p53, resulting in deregulated cell cycle arrest upon DNA damage and an inhibition of apoptosis.[66] E7 inactivates pRb and releases E2F transcription factors, causing increased proliferation.[67] The disruption of cell cycle control and apoptosis, often accompanied by viral DNA integration resulting in viral persistence, contributes to the onset of carcinogenesis.[65]

An unifocal VIN is believed to be caused by only one HPV genotype. If multiple HPV genotypes are detected, one HPV type is a bystander, while the other type caused the lesion.[68]

DNA methylation of host-cell genes

DNA methylation is a common epigenetic event and an important regulator of gene expression.[69] Methylation involves the addition of a methyl group (CH3) to a cytosine in a cytosine-guanine dinucleotide (CpG).[70] Increased methylation (hypermethylation) of CpG-rich regions in the promoter of tumor suppressor genes, can lead to transcriptional repressionand subsequent loss of tumor suppressive

function (Figure 6).[71] Hypermethylation of tumor suppressor gene promoters can be induced by activation of the DNA methylation machinery through the viral oncoproteins E6 and E7.[72] The loss of tumor suppressive function contributes to the development of cancer. Quantitative methylation-specific PCR (qMSP) is an objective and sensitive method to analyze the DNA methylation levels of specific genes. DNA methylation can be assessed by qMSP in various sample types, e.g. paraffin-embedded tissue, blood, scrapes and urine. Nowadays, identification of methylation changes have emerged as a promising biomarker for diagnosis, prognosis and prediction of many tumor types.

In HPV-related anogenital diseases, DNA methylation tests hold promise for identifying precursors with high cancer risk.[73, 74] Various methylation markers associated with HPV-induced anogenital carcinogenesis have been discovered by our group, of which *ASCL1, CADM1, FAM19A4, GHSR, LHX8, MAL, miR124-2, PHACTR3, PRDM14, SST, ZIC1* and *ZNF582* were used to assess the potential value for cancer risk prediction in patients with VIN, as described in this thesis.[75-77]

Immunohistochemistry

P16^{INK4A}

P16^{INK4a} immunohistochemistry is a reliable surrogate marker for hr-HPV infection. Expression of p16^{INK4a} is the result of cell cycle deregulation induced by hr-HPV E7 viral oncogene activity via binding of E7 to pRB.[78] Binding of E7 to pRB results in upregulated E2F activity and p16^{INK4a} protein due to disruption of the negative feedback loop.[79] According to the Lower Anogenital Squamous Terminology (LAST) Project, a positive p16^{INK4a} staining pattern is diffuse, strong and continuous (nuclear and cytoplasmic), referred to as a 'block-like', in at least the lower one-third of the epithelium.[80] Almost 100% of HSIL are block-positive for p16^{INK4a} (Figure 4C), as well as 4 to 20% of LSIL.[64, 81-83]

P53

The *TP53* gene is a tumor suppressor gene and the p53 protein functions as a transcription factor in the nucleus. In response to various stresses, such as DNA damage and oncogenic signaling, *TP53* induces cell cycle arrest to allow repair, or when DNA damage is beyond repair, to initiate apoptosis. In HPV-independent VIN, the *TP53* gene is often mutated and p53 aberrant or mutant staining is found in approximately 70% of cases (Figure 5C). TP53 mutations are very uncommon in HSIL.[84] P53 wild-type staining can be categorized as i) scattered, weak of very weak nuclear staining (respectively 'conventional' and 'reduced, mimicking a null-mutant

pattern'), or ii) mid-epithelial staining with basal-sparing. P53 mutant staining is categorized as: i) (para)basal/diffuse staining, ii) absent (null pattern) staining, and iii) cytoplasmic staining.[85, 86]

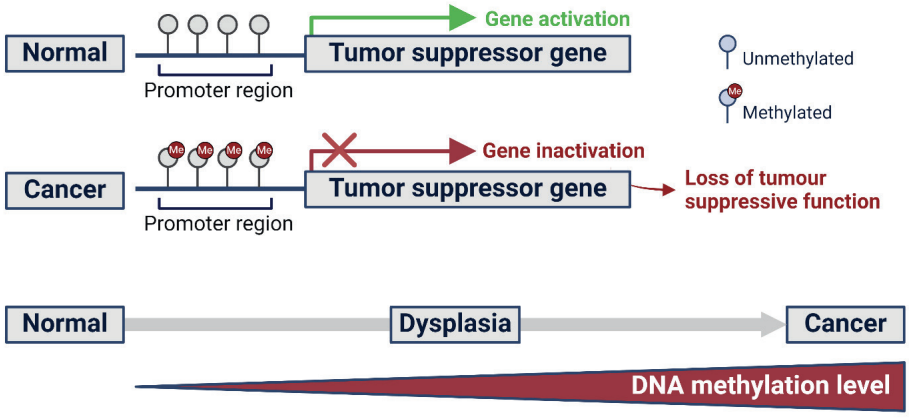


Figure 6. Host cell DNA methylation-mediated silencing of tumor suppressor genes. Created with Biorender.com.

Ki-67

In the assessment of VIN lesions, immunohistochemical staining for $p16^{INK4a}$ or p53 is often combined with staining for Ki-67. Ki-67 is a marker of cellular proliferation that is weakly, scattered expressed in the proliferating parabasal nuclei of cells in healthy vulvar skin epithelium, whereas in VIN, expression can be observed in all viral induced or dysplastic cells.[87, 88]

CK17 and SOX2

Recent studies support the use of immunohistochemical markers CK17 and SOX2 for the classification of high-grade VIN as adjunct to morphology and established markers $p16^{INK4a}$ and p53. CK17 is an intermediate filament protein induced in activated keratinocytes.[89] SOX2 (Sex-determining region Y-box 2) is located on chromosomal segment 3q26.33 and is an important regulator of pluripotent stem cells promoting maintenance and development of the squamous epithelium.[90] Both CK17 and SOX2 have been reported to show increased expression in VIN and other neoplasia compared to normal or reactive tissues.[41, 91-93]

Thesis outline

To improve care for patients with VIN, a better understanding of the vulvar cancer risk is needed. This thesis aims to investigate the potential of objective biomarkers for cancer risk stratification of VIN patients.

Towards the goal of accurate cancer risk assessment, a longitudinal, population-based historical cohort series including 1,148 patients with an original diagnosis of high-grade VIN is studied in **Chapter 2**. In this chapter, the incidence of VIN is calculated, stratified for HSIL and HPV-independent VIN (dVIN). In addition, vulvar cancer risk and associated risk factors are studied in the 894 patients without concurrent vulvar cancer at baseline. Given the low number of studies on HPV-independent VIN, a systematic literature review is performed (**Chapter 3)**, investigating the primary and recurrent risk of progression to vulvar cancer in patients with HPV-independent VIN (dVIN).

In **Chapter 4**, twelve candidate methylation markers associated with HPV-induced anogenital carcinogenesis are evaluated in a cross-sectional pilot series of 192 vulvar tissue samples, including healthy vulvar tissues and both HPV-associated and HPV-independent (pre-)cancers. Those lesions represent a wide spectrum of vulvar lesions. To further explore the heterogeneity of biomarker expression in patients with multifocal HSIL, methylation profiles, HPV genotype and IHC expression of $p16^{INK4a}$ and Ki-67 are studied in a pilot series of 32 vulvar lesions from 12 patients (**Chapter 5)**.

In **Chapter 6**, 751 high-grade VIN from patients without previous or concurrent vulvar cancer from the historical cohort described in Chapter 2, are comprehensively characterized and categorized in HPV-associated and HPV-independent vulvar lesions. The importance and the diagnostic utility of $p16^{INK4a}$, p53 and Ki-67 immunohistochemical markers and HPV genotyping are presented, and stratified vulvar cancer risks are given. In **Chapter 7,** we cross-sectionally validate the 12 DNA methylation markers in the 751 vulvar tissue samples of the historical cohort described in Chapter 6. Methylation levels in relation to disease category are studied and the best performing three-gene marker panel for detection of high-grade VIN is determined. In **Chapter 8**, both the optimal three-gene methylation marker panel as determined in Chapter 7 and other risk factors in relation to cancer risk, are evaluated in all 578 HSIL and 46 HPV-independent VIN patients from the historical cohort, as identified in Chapter 6.

In **Chapter 9** the performance of immunohistochemical markers CK17 and SOX2 is validated in a series of 150 vulvar lesions from the historical cohort described in Chapter 6. These 150 cases are reviewed by six experts in vulvar pathology.

In **Chapter 10**, the main findings of this thesis are discussed and related to current and future perspectives.

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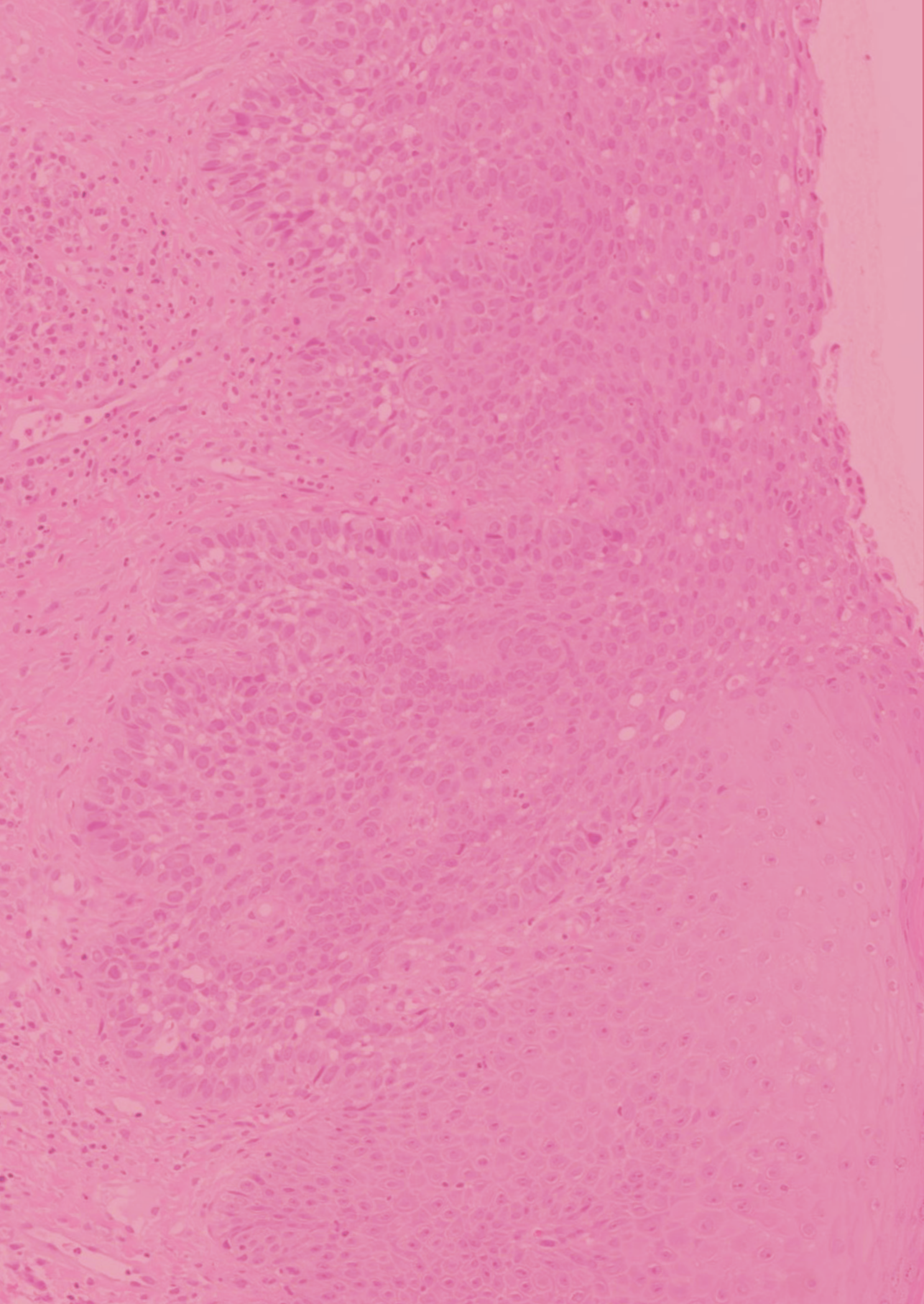

CHAPTER 2

Vulvar intraepithelial neoplasia: incidence and long-term risk of vulvar squamous cell carcinoma

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Abstract

The risk of vulvar squamous cell carcinoma (VSCC) in patients with high-grade vulvar intraepithelial neoplasia (VIN) is considered lower in high-grade squamous intraepithelial lesion (HSIL) compared to differentiated VIN (dVIN), but studies are limited. This study investigated both the incidence of high-grade VIN and the cumulative incidence of VSCC in patients with HSIL and dVIN separately.A database of women diagnosed with high-grade VIN between 1991 and 2011 was constructed with data from the Dutch Pathology Registry (PALGA). The European standardized incidence rate (ESR) and VSCC risk were calculated, stratified for HSIL and dVIN. The effects of type of VIN (HSIL versus dVIN), age and lichen sclerosis (LS) were estimated by Cox regression.In total, 1,148 patients were diagnosed with high-grade VIN between 1991-2011. Between 1991-1995 and 2006-2011, the ESR of HSIL increased from 2.39 (per 100,000 woman-years) to 3.26 and the ESR of dVIN increased from 0.02 to 0.08. The 10-year cumulative VSCC risk was 10.3%; 9.7% for HSIL and 50.0% for dVIN (log rank $p<0.001$). Type of VIN, age and presence of LS were independent risk factors for progression to VSCC, with hazard ratios of 3.0 (95% CI 1.3-7.1), 2.3 (95% CI 1.5-3.4) and 3.1 (95% CI 1.8-5.3) respectively.The incidence of high-grade VIN is rising. Because of the high cancer risk in patients with dVIN, better identification and timely recognition are urgently needed.

Introduction

Vulvar squamous cell carcinoma (VSCC) accounts for more than 90% of all vulvar cancers.[1] The etiology of these tumors is recognized to be diverse.[2, 3] About 15-25% of the VSCCs are induced by high-risk human papillomavirus (HPV), whereas the majority of VSCCs are HPV-negative and associated with lichen sclerosus (LS).[4-8]

VSCC develops from precursor lesions, covered by the term high-grade vulvar intraepithelial neoplasia (VIN). The 2015 International Society for the Study of Vulvovaginal Disease (ISSVD) terminology of vulvar squamous intraepithelial lesions classifies high-grade VIN into high-grade squamous intraepithelial lesion (HSIL) and differentiated VIN (dVIN).[9] Studies have shown that most patients with high-grade VIN are diagnosed with HSIL, and in 75 to 85% of HSIL lesions HPV positivity has been demonstrated.[4, 8, 10] On the contrary, dVIN is only diagnosed in a small subset of patients with high-grade VIN, is independent of HPV and is associated with the presence of LS.

In this study, we aimed to estimate (1) the incidence of high-grade VIN diagnosed between 1991 and 2011 in the Netherlands, and (2) the long-term VSCC incidence in patients with high-grade VIN, stratified for HSIL and dVIN.

Materials and Methods

Study design, data collection and study population

For this study, women diagnosed with high-grade VIN were selected from a historical cohort. Detailed characteristics of this historical cohort have been described previously.[11] In short, a database was constructed with data from the nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA), which reached nationwide coverage in 1991. All vulvar pathology reports of patients with a diagnosis of lichen sclerosus (LS), vulvar intraepithelial neoplasia (VIN) and/or vulvar squamous cell carcinoma (VSCC) diagnosed up to June 2011 were collected. To obtain a dataset reflecting a representative set of the Dutch female population, pathology data of all laboratories in the provinces Noord-Holland and Flevoland were selected, because these laboratories supply the regional collaborating hospital network, including referral centers and the 3 centers of gynecologic oncology in Amsterdam. The provinces Noord-Holland and Flevoland are situated in the North-West of the Netherlands and represented 17.4% to 18.7% of the female population in the Netherlands between 1991 and 2011.[12] Since

nationwide coverage of PALGA was obtained in 1991, only patients with incident high-grade VIN diagnosed thereafter were included in this study. From this cohort, additional follow up data up to 2018 were collected. All 18,604 pathology reports were reviewed to categorize the pathology results. Patients with high-grade VIN were excluded from the analyses when they had a history of VSCC.

Classification of high-grade VIN

All high-grade VIN cases were classified into HSIL or dVIN, based on the diagnosis in the pathology report and according to the 2015 ISSVD terminology. HSIL was termed "vulvar intraepithelial neoplasia usual type" (uVIN) in the 2004 ISSVD terminology, 'squamous intraepithelial lesion' (SIL) in the 1994 World Health Organization (WHO) terminology and 'VIN2' or 'VIN3' in the 1989 WHO terminology. Therefore, HSIL included the following diagnosis: usual type of VIN, morbus Bowen, bowenoid papulosis, erythroplasia of Queyrat, VIN2, VIN3, high-grade VIN (not otherwise specified) and carcinoma in situ. DVIN included, in addition to dVIN, also vulvar dystrophy with atypia and simplex VIN.

Presence of LS

Histopathological diagnoses lichen sclerosus and possible lichen sclerosus were both categorized as lichen sclerosus, as previously described.[11] Possible lichen sclerosus included cases with interface dermatitis that could fit with an early phase of lichen sclerosus. Only biopsy proven (possible) vulvar LS reported prior to the diagnosis of high-grade VIN or within an interval of 3 months after incident VIN diagnosis, was included.

Statistical analysis

Incidence of high-grade VIN

The crude incidence rate of high-grade VIN was calculated from the number of patients diagnosed with high-grade VIN. The total number of woman-years was calculated from the female population in Noord-Holland and Flevoland (retrieved from Statistics Netherlands)[12]. The European Standard Population (2013) was used to calculate the European Standardized Rate (ESR). Calendar year at time of diagnosis was stratified into the periods 1991-1995, 1996-2000, 2001-2005 and 2006-2011. Because a subgroup of patients with high-grade VIN was diagnosed with concurrent VSCC, analyses were performed with and without cases with concurrent VSCC. High-grade VIN with concurrent VSCC was defined as a diagnosis of VSCC within 3 months from VIN diagnosis.

Risk of VSCC in patients with VIN

The incidence rate of VSCC per 100,000 woman-years at risk was calculated among patients with high-grade VIN without concurrent VSCC. The Kaplan-Meier method was used to adjust for censoring. Follow-up time was calculated from the date of the first histological diagnosis of high-grade VIN to the date of the first histological diagnosis of VSCC. Patients who did not develop VSCC had an end date set equal to the earliest date of either their expected date of death or the date of data extraction from PALGA. The expected date of death was retrieved from age-dependent life expectancy tables of Statistics Netherlands at the time of the last vulvar pathology report.[12]

Differences between Kaplan-Meier curves were evaluated by log-rank tests. Multiple Cox regression analyses and Wald tests were performed to assess the effects of multiple risk factors. Median age in different strata were compared by Mann-Whitney U or Kruskal-Wallis Tests. The level of statistical significance was set at 0.05. Statistical analysis was performed using IBM SPSS Statistics software for Windows version 24.0 (IBM Corporation, Armonk, NY).

Results

Characteristics of the study population

The baseline characteristics of the study population are presented in Table 1. Between 1991 and 2011, 1,148 patients were diagnosed with incident high-grade VIN, comprising 1,116 (97.2%) patients with HSIL and 32 (2.8%) patients with dVIN.

Biopsy proven LS was present in 112/1,148 (9.8%) patients with high-grade VIN. LS was more common in patients with dVIN (14/32; 43.8%) than in patients with HSIL (98/1,116; 8.8%, $p<0.001$).

Concurrent VSCC was seen in 254 (22.1%) patients with high-grade VIN and was more often seen in patients with dVIN (62.5%) than in patients with HSIL(21.0%, $p<0.001$).

The total number of patients diagnosed with high-grade VIN increased by calendar period, from 188 incident cases between 1991-1995 to 385 incident cases between 2006-2010. The number of newly diagnosed patients increased between 1991-1995 and 2006-2010 from 187 to 367 for HSIL and from 1 to 18 patients for dVIN.

Median age at time of high-grade VIN diagnosis was 49.8 years and ranged from 16.1 to 95.4 years. The median age was significantly higher in patients with dVIN (70.3 years) compared to patients with HSIL (49.2 years, p<0.001), as well as in patients with concurrent VSCC (68.7 years) compared to patients without concurrent VSCC (45.7 years, p<0.001). Median age at time of high-grade VIN diagnosis increased by calendar period, from 44.9 years between 1991-1995 to 53.2 years between 2006-2011 (p<0.001).

Table 1. Baseline characteristics of the study population

	n	%	Age, median	(range)	p
High-grade VIN	1.148	100	49.8	(16.1-95.4)	
HSIL	1.116	97.2	49.2	(16.1-95.4)	
dVIN	32	2.8	70.3	(40.3-85.3)	<0.001
Lichen sclerosus					
no	1.036	90.2	48.3	(17.4-95.4)	
yes	112	9.8	68.5	(16.1-91.5)	<0.001
Concurrent VSCC					
no	894	77.9	45.7	(16.3-92.3)	
yes	254	22.1	68.7	(30.0-95.4)	<0.001
Period					
1991-1995	188	16.4	44.9	(16.1-92.5)	
1996-2000	247	21.5	45.2	(17.8-91.5)	
2001-2005	296	25.8	49.7	(19.6-93.9)	
2006-2011	417	36.3	53.2	(20.3-95.4)	<0.001

Abbreviations: VIN = high-grade vulvar intraepithelial neoplasia, HSIL = high grade squamous intraepithelial lesion, dVIN = differentiated VIN, VSCC = vulvar squamous cell carcinoma.

Incidence of high-grade VIN

The crude incidence rates of high-grade VIN with and without concurrent VSCC in relation to age are shown in Figure 1. The incidence rate of high-grade VIN without concurrent VSCC showed a peak of 5.1 per 100,000 woman-years between the age of 35 and 40 (Figure 1A, continuous line). The incidence of patients with high-grade VIN including patients with concurrent VSCC, showed a peak incidence of 7.6 between the age of 85 and 89 (Figure 1A, interrupted line).

Stratification for HSIL and dVIN (Figure 1B) revealed that incidence rates of HSIL were very similar to those of high-grade VIN, reflecting the large overlap between the two

groups. In contrast, the incidence rates of dVIN had a different pattern, with a disease onset after the age of 50 years and with most women diagnosed with concurrent VSCC (Figure 2B).

The ESRs and crude incidence rates of high-grade VIN with and without concurrent VSCC are displayed in Table 2A and 2B, respectively. Overall, the ESR of high-grade VIN without concurrent VSCC was 2.99 per 100,000 woman-years; 2.95 for HSIL and 0.05 for dVIN (Table 2B). The ESR increased from 2.41 in period 1991-1995 to 3.33 in period 2006-2011 (+38.2%); from 2.39 to 3.26 (+36.4%) for HSIL and from 0.02 to 0.08 (+300.0%) for dVIN. The ESR of high-grade VIN including concurrent VSCC was 3.97 per 100,000 woman-years; 3.85 for HSIL and 0.13 for dVIN (Table 2A). The ESR increased from 2.87 in period 1991-1995 to 4.75 in period 2006-2011 (+65.5%); from 2.87 to 4.46 (+55.4%) for HSIL and from 0.02 to 0.28 (+1,300.0%) for dVIN.

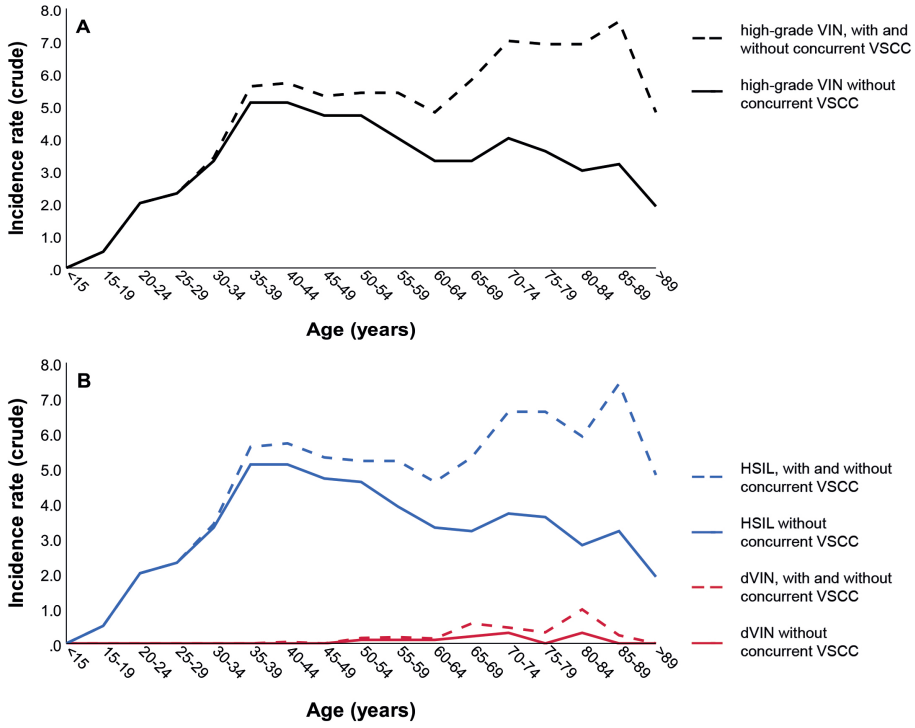
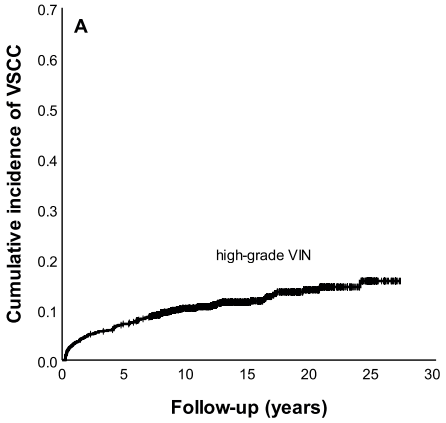
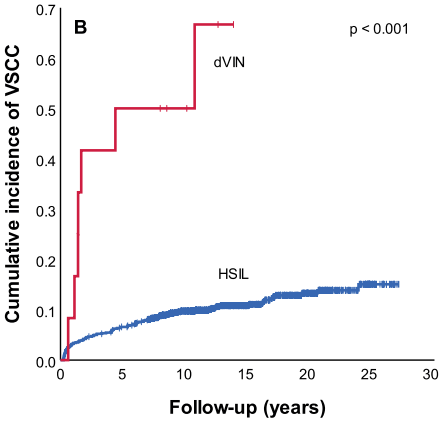


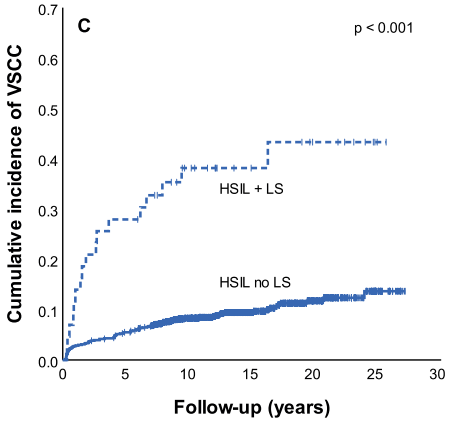
Figure 1. A, All high-grade VIN. B, high-grade VIN stratified for HSIL (blue line) and dVIN (red line). Interrupted lines represent VIN, both with and without concurrent VSCC. Continuous lines include VIN without concurrent VSCC. dVIN, differentiated VIN; HSIL, high-grade squamous intraepithelial lesion; VIN, vulvar intraepithelial neoplasia; VSCC, vulvar squamous cell carcinoma



Cum. Incidence of VSCC (%)	7.2	10.3	11.5	14.0	15.7	
No. of remaining cases	894	824	630	367	184	46



dVIN		50.0	50.0	66.7*		
HSIL		6.6	9.7	10.8	13.3	15.1
dVIN	12	6	4	0*		
HSIL	882	818	626	367	184	46



HSIL + LS		27.9	38.1	38.1	43.3	43.3
HSIL no LS		5.5	8.3	9.4	11.7	13.6
HSIL + LS	43	31	20	13	9	2
HSIL no LS	839	787	606	354	175	44

Figure 2. A, All high-grade VIN. B, High-grade VIN stratified for HSIL (blue line) and dVIN (red line). C, HSIL stratified for presence of LS (interrupted line) and absence of LS (continuous line). *after 14 years of follow-up. dVIN, differentiated VIN; HSIL, high grade squamous intraepithelial lesion; LS, lichen sclerosus; VSCC, vulvar squamous cell carcinoma.

Table 2. European standardized rate (ESR) and crude incidence rate of high-grade vulvar intraepithelial neoplasia (VIN) per 100,000 woman-years between 1991 and 2011

A	high-grade VIN (n = 1.148)					HSIL					dVIN				
Age (years)	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**
<30	0.89	0.93	0.94	0.94	0.77	0.89	0.93	0.94	0.94	0.77	0.00	0.00	0.00	0.00	0.00
30-49	5.01	4.00	5.25	5.25	5.36	5.00	4.05	5.25	5.21	5.33	0.01	0.00	0.00	0.04	0.00
50-69	5.33	3.38	4.17	5.53	7.09	5.09	3.29	4.17	5.34	6.57	0.24	0.08	0.00	0.19	0.52
≥70	6.92	4.69	5.83	7.40	9.08	6.46	4.69	5.71	6.91	8.00	0.47	0.00	0.12	0.49	1.07
All ages															
Incidence crude	3.77	2.75	3.49	4.00	4.54	3.70	2.70	3.50	3.90	4.30	0.11	0.02	0.01	0.11	0.24
Incidence ESR	3.97	2.87	3.65	4.20	4.75	3.85	2.87	3.63	4.07	4.46	0.13	0.02	0.02	0.13	0.28

B	high-grade VIN (n = 894)					HSIL					dVIN				
Age (years)	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**	**'91-'11**	**'91-'95**	**'96-'00**	**'01-'05**	**'06-'11**
<30	0.89	0.93	0.94	0.94	0.77	0.89	0.93	0.94	0.94	0.77	0.00	0.00	0.00	0.00	0.00
30-49	4.53	3.91	4.85	4.53	4.72	4.52	3.91	4.85	4.49	4.72	0.01	0.00	0.00	0.04	0.00
50-69	3.92	2.49	3.44	4.05	4.96	3.82	2.41	3.44	3.93	4.82	0.10	0.08	0.00	0.13	0.14
≥70	3.45	3.12	2.23	4.12	4.10	3.30	3.12	2.23	3.88	3.81	0.15	0.00	0.00	0.24	0.29
All ages															
Incidence crude	2.93	2.38	2.81	3.09	3.30	2.90	2.40	2.80	3.00	3.20	0.04	0.02	0.00	0.07	0.07
Incidence ESR	2.99	2.41	2.84	3.16	3.33	2.95	2.39	2.84	3.08	3.26	0.05	0.02	0.00	0.08	0.08

A. High-grade VIN, total group with and without concurrent VSCC. B. High-grade VIN without concurrent VSCC.

Table 3. Prognostic factors for vulvar squamous cell carcinoma (VSCC) in women with high-grade vulvar intraepithelial neoplasia (VIN)

	UNIVARIABLE ANALYSIS												MULTIVARIABLE ANALYSIS			
	high-grade VIN				HSIL				dVIN				high-grade VIN			
	n	HR	(95% CI)	p	n	HR	(95% CI)	p	n	HR	(95% CI)	p	n	HR	(95% CI)	p
Type of VIN																
HSIL	882	1.0											882	1.0		
dVIN	12	8.2	3.8-17.7	<0.001									12	3.0	1.3-7.1	0.013
Age (years)																
<50	530	1.0			529	1.0			1	1.0			530	1.0		
≥50	364	2.3	1.5-3.4	<0.001	353	2.5	1.7-3.8	<0.001	11	3.3	0.0-3.510	0.146	364	2.3	1.5-3.4	<0.001
Lichen sclerosus																
no	845	1.0			839	1.0			6	1.0			845	1.0		
yes	49	5.2	3.2-8.4	<0.001	43	4.8	2.9-8.1	<0.001	6	1.2	0.3-5.6	0.782	49	3.1	1.8-5.3	<0.001
Period																
1991-1995	162	1.0			161	1.0			1	1.0			162	1.0		
1996-2000	199	0.7	0.4-1.2	0.205	199	0.7	0.4-1.3	0.252	0	-			199	0.8	0.5-1.4	0.394
2001-2005	229	0.7	0.4-1.3	0.288	224	0.7	0.4-1.2	0.198	5	0.0	0.1-9.6	0.981	229	0.8	0.4-1.3	0.328
2006-2011	304	0.8	0.5-1.3	0.342	198	0.7	0.4-1.3	0.260	6	0.0	0.1-10.9	0.999	304	0.8	0.4-1.3	0.336

Cox regression analysis was performed to calculate the adjusted hazard ratio (HR) and 95% confidence interval (CI). Adjustments were made for all factors in the table. Statistical significance is presented in bold.

Incidence of VSCC in patients with high-grade VIN

To analyze the incidence rate of VSCC in patients with high-grade VIN, 254 patients with concurrent VSCC were excluded from the analysis. The remaining 894 patients had a median follow-up time of 13.9 years (range 0.3-27.4 years), with a total of 12,435 woman-years available for analyses. The incidence rate of VSCC was 861 per 100,000 woman-years. During follow-up, 107/894 (12.0%) patients were diagnosed with incident VSCC; 100/882 (11.3%) patients with HSIL and 7/12 (58.3%) patients with dVIN. Median progression time to VSCC was 4.0 years (ranging from 0.3 to 24.2 years) after high-grade VIN diagnosis; 4.1 years for HSIL and 1.4 years for dVIN, which was not significant (p=0.449).

The cumulative incidence of VSCC is shown in Figure 2. In patients with high-grade VIN, the cumulative VSCC incidence after 27.4 years was 15.7% (95% confidence interval (CI), 12.0-19.4%). The cumulative VSCC incidence increased rapidly the first five years and more or less linear thereafter (Figure 2A); after 5 years the cumulative incidence was 7.2% (95% CI, 5.4-9.0%), after 10 years 10.3% (95% CI, 8.3-12.3%), after 15 years 11.5% (95% CI, 9.3-13.7%) and after 20 years 14.0% (95% CI, 11.3-16.7%).

In patients with dVIN, the 10-year cumulative VSCC incidence was much higher (50.0%, 95% CI 21.8-78.2%) than in patients with HSIL (9.7%, 95% CI 7.7-11.7%), p<0.001, Figure 2B). Patients with HSIL and LS had a significantly higher 10-year cumulative VSCC incidence compared to patients with HSIL without LS, respectively 38.1% (95% CI, 23.2-53.0%) versus 8.3% (95% CI, 6.3-10.3, log rank p<0.001, Figure 2C).

Univariate Cox regression analysis of type of high-grade VIN, age at time of VIN diagnosis, LS and calendar period showed that type of VIN, age and LS were independent risk factors for VSCC (Table 3). Patients with dVIN had a 8.2 times higher cancer risk than patients with HSIL. Patients with an age of 50 years or older at time of VIN diagnosis had a 2.3 times higher cancer risk than patients under the age of 50 years, and patients with LS had a 5.2 times higher cancer risk than patients without LS. Corrected for all variables in the multivariate cox regression analysis, the variables type of VIN, age and LS remained independent risk factors for VSCC (Table 3), with hazard ratios of respectively 3.0, 2.3 and 3.1.

Discussion

In this unique, large series of patients with high-grade VIN, we observed an increased incidence over time and a 10-year cumulative vulvar cancer risk of 10.3%, which was highly dependent on type of VIN, presence of LS and age at diagnosis. Our study on 1,148 women with high-grade VIN demonstrated a much higher cancer risk of 50.0% in patients with dVIN compared to a risk of 9.7% in patients with HSIL after 10 years of follow-up.

Studies on the vulvar cancer risk in patients with VIN are scarce. A 5-year cumulative cancer risk of 0% was found in one study, including only 18 patients with HSIL.[13] Absolute cancer risks have been reported slightly more often, ranging 2.3 to 6.6% after an average follow-up time of 3 years.[14-21] Consistent with these findings, we found a 5-year cumulative cancer risk of 6.6.% and an absolute cancer risk of 5.7% after 3 years in our series of 882 patients with HSIL. The stable vulvar cancer risk over time found in our study, makes life-long surveillance of patients with HSIL necessary. Of note, the reported cancer cases reflect outcome after treatment, meaning that the risk of invasive cancer in patients with untreated VIN is likely to be higher.

While dVIN is considered to be more aggressive than HSIL, cancer risks have been assessed only in a limited number of studies.[13, 19, 22, 23] Consistent with the aggressive nature of dVIN, we found an absolute cancer risk of 58% in 12 patients with dVIN after 14 years of follow-up. In another small series of 7 patients with dVIN, an absolute cancer risk of 86% after 6 years was reported.[13] A larger study including 67 patients with dVIN found an absolute cancer risk of 33% after 14 years of follow-up.[19] However, in this latter study, dVIN also included patients with high-grade VIN in combination with LS or a negative HPV test result.[19] This definition of dVIN might bias the results as the occurrence of high-grade VIN and LS can coexist independently. The aggressive nature of dVIN might be explained by a relative short intraepithelial phase before progression to invasive carcinoma. This is supported by our study in which the interval to carcinoma was 1.4 years for dVIN and 4.1 years for HSIL, although this difference was not statistically significant. The malignant potential of dVIN was also reflected by the high number of patients with dVIN presenting with concurrent VSCC, which was 62.5%, compared to 21.0% in patients with HSIL.

In our study, only 32 (2.8%) of all 1,148 high-grade VIN cases were reported as dVIN, which is consistent with the low prevalence described by others.[19, 24] Because dVIN is often difficult to recognize for patients as well as for clinicians, including pathologists, it may partly explain why so few patients have been diagnosed with

dVIN.[23, 25, 26] Signs of dVIN can be variable and often subtle, leading to misdiagnoses and inadequate clinical care due to diagnostic delay, especially in centers with limited exposure to this rare disease.[27-29] It has been shown that dVIN was missed in 42% of biopsies initially diagnosed as LS in a series of patients who developed VSCC.[30] The current classification dividing high-grade VIN into HSIL and dVIN is morphology-based rather than biologically-defined, but not all HPV-independent VIN have a dVIN morphology.[31-33] HPV status of the VIN lesions was not systematically examined during regular care in our study cohort. Consequently, the influence of HPV status on the clinical course could not be adequately investigated. Additional studies are needed to investigate whether a biologically-defined classification in HPV-induced and HPV-independent VIN with the use of both morphology and laboratory tests can lead to better categorization of patients with high-grade VIN.

In addition to type of VIN, presence of LS and higher age also proved to be important risk factors for vulvar cancer development in our study. Altered immunity could explain the higher incidence of VSCC in patients with VIN and LS and in elderly patients with VIN, although it has never been confirmed that vulvar LS is an autoimmune condition.[34, 35] Furthermore, longer-standing, untreated VIN lesions at time of diagnosis in older patients could account for the high cancer risk in this patient group. Interestingly, we noted an incidence of LS of 8.8% in patients with vulvar HSIL, which is high compared to the estimated incidence of 1.5%–2.5% in the general or gynecologic population.[11, 36, 37] Dysregulated immunity could be a possible explanation for the coexistence of LS and HSIL. Alternatively, patients with HSIL and LS might in fact have HPV-independent high-grade VIN with the same aggressive course as dVIN. Further research investigating detailed information of clinicopathological aspects, including HPV status, is needed to clarify the relationship between HSIL and LS.

In our study, we observed an incidence of high-grade VIN of 3.8 per 100,000 women-years, which corresponds to incidences reported in the literature (i.e. 0.23 to 5.0 per 100,000 woman-years).[38-40] Also in line with others, we observed an increased incidence of +38.2% in our 20-year study period.[15, 40] There are several plausible explanations for the rising incidence of high-grade VIN. First, aging of the population could have led to more VIN diagnoses in elderly patients. This is supported by the increased incidence of high-grade VIN in older age groups as observed in our study cohort. Second, an increased burden of HPV-related disease could have contributed to the rising incidence of VIN.[38-40] Of note, as VIN was diagnosed in our study cohort in the pre-vaccination era, no effect of HPV vaccination was expected. However, with second generation HPV vaccination, virtually all cases of vulvar HSIL are potentially

preventable in the coming decades.[41-43] Third, vulvar pathology has gained more public and clinical awareness, which subsequently could have led to more clinical visits and vulvar biopsies in patients with VIN.[44]

One of the strengths of our study is the large study size of 1,148 patients with VIN, which is a high number given the rarity of the disease. Consequently, we were able to study HSIL and dVIN separately, thereby providing new evidence that HSIL and dVIN are two distinct disease entities. Second, selection bias of our study cohort was limited by the use of data covering a well-identified region, instead of the use of institutional data, making our study results representative for the general population. Lastly, accurate long-term cancer risk in patients with VIN could be estimated because long-term follow-up data up to 27.4 years were available.

This study also has some limitations. Our results are primarily based on reported long-term pathology data without additional revision of the pathology slides. Since the classification of VIN has been changed over time and awareness of the dVIN entity was limited in the early study period, revision of the pathology slides could have resulted in more accurate categorization into HSIL and dVIN. In addition, limited clinical data were available. Only information on biopsy proven LS was available, thereby missing clinically diagnosed LS. Alternatively, LS might have been underreported when co-existing next to dVIN tissue.

In conclusion, high-grade VIN is a heterogeneous disease comprising two different disease entities, with a rising incidence. An alarmingly higher cancer risk and shorter interval to cancer was found in patients with dVIN compared to patients with HSIL. Earlier and more adequate identification of these precursor lesions with high cancer risk is therefore of utmost importance. In contrast to dVIN, the cancer risk of HSIL is relatively low, except for when LS is present. Hence, patients with HSIL could benefit from risk stratification to reduce overtreatment. Molecular biomarkers that could identify dVIN at an early stage and that could cancer risk stratify HSIL are therefore highly needed.[45, 46]

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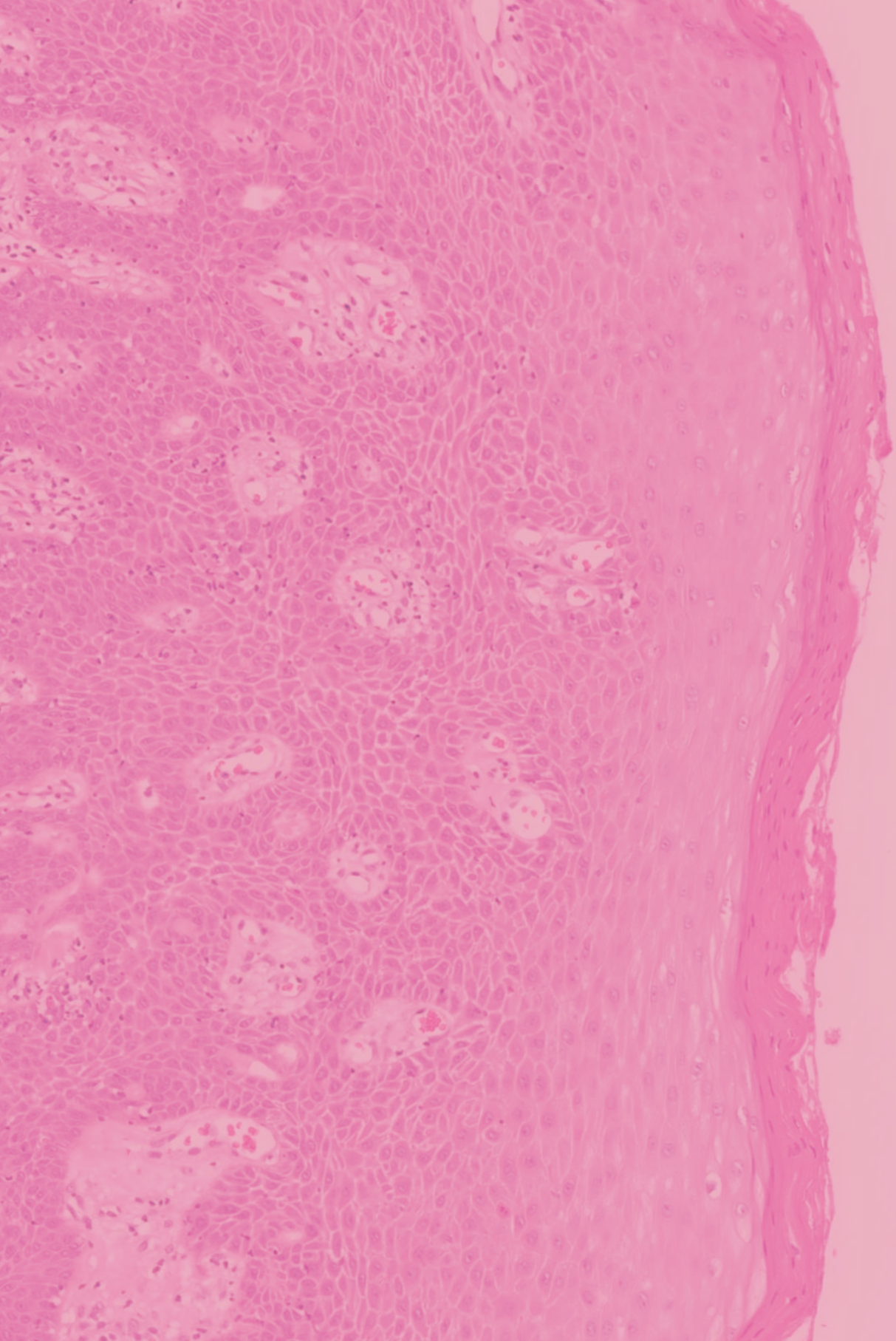

CHAPTER 3

The Vulvar Cancer Risk in Differentiated Vulvar Intraepithelial Neoplasia: A Systematic Review

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Abstract

Differentiated vulvar intraepithelial neoplasia (dVIN) is the precursor of human papillomavirus (HPV)-independent vulvar squamous cell carcinoma (VSCC). Given the rare incidence of dVIN, limited information on the exact cancer risk is available. We systematically reviewed the primary and recurrent VSCC risk in patients with dVIN, as well as the time to cancer development. A systematic search was performed up to July 2021 according to the PRISMA guidelines. Five reviewers independently screened articles on title, abstract and full text, followed by critical appraisal of selected articles using the Quality in Prognostic Studies (QUIPS) tool. Of the 455 screened articles, 7 were included for analysis. The absolute risk for primary VSCC in dVIN varied between 33 and 86%, with a median time to progression to VSCC of 9–23 months. The risk of developing recurrent VSCC in dVIN associated VSCC was 32–94%, with a median time to recurrence of 13–32 months. In conclusion, patients with dVIN have a high risk of developing primary and recurrent VSCC with a short time to cancer progression. Increased awareness, timely recognition, aggressive treatment and close follow-up of HPV-independent vulvar conditions including dVIN is therefore strongly recommended.

Introduction

Vulvar intraepithelial neoplasia (VIN) is the precursor lesion of vulvar squamous cell carcinoma (VSCC), which is categorized into human papillomavirus (HPV)-induced vulvar high-grade squamous intra-epithelial lesions (vHSIL) and HPV-independent differentiated VIN (dVIN).(1-5) The vast majority of high-grade VIN lesions are diagnosed as vHSIL, with a known cancer risk of 3–10%.(6-9) DVIN comprises less than 5% of VIN lesions.(10) Nevertheless, the majority of VSCC are HPV-independent, indicating the high malignant potential of dVIN.(1, 2, 5)

After the first dVIN case was described by Abell and Gosling in 1961 as an 'intraepithelial carcinoma of simplex type', it was not until 1986 that the terminology 'differentiated VIN' was adopted by the International Society for the Study of Vulvovaginal Disease (ISSVD).(11, 12) In 2004 the ISSVD proposed a classification scheme distinguishing HPVinduced high-grade vulvar neoplasia, formerly known as usual type VIN (nowadays vHSIL), from HPV-independent dVIN.(13)

Besides the absence of HPV infection, dVIN has several other clinical and pathologic features that distinguish it from the more frequently diagnosed vHSIL. DVIN almost always arises in a background of lichen sclerosus (LS), a chronic inflammatory dermatosis which mainly occurs in the anogenital area of postmenopausal women. Both LS and dVIN typically occur in women above the age of 60 years, although it can affect younger women as well.(14)

Although dVIN represents the minority of diagnosed VIN lesions, it is often diagnosed adjacent to VSCC.(8) DVIN is therefore regarded as the more aggressive precursor lesion when compared to vHSIL and other non-neoplastic epithelial disorders, such as LS and lichen planus.(14) Given the aggressive nature of dVIN, it is currently receiving increasing attention concerning methods to improve diagnostics and clinical management. Solitary dVIN is a rare finding and difficult to diagnose due to its varying presentation. Therefore, limited information on the exact cancer risk is available. As a result, current treatment of dVIN, consisting of surgical excision, is based on small case series and expert opinions.

The purpose of the present study was to review current literature on the risk of developing primary and recurrent VSCC in patients with dVIN, including the time to cancer progression. Better insight in the cancer risk can create a more scientific basis for an evidence-based guideline for the treatment of dVIN.

Materials and Methods

The methods and results are reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.(15) This study was registered with Research Registry and the unique identifying number is: reviewregistry 1243 (https://www.researchregistry.com, accessed on 1 November 2021).

Search Strategy

To identify all relevant publications on cancer risk in dVIN, we performed a systematic electronic search in bibliographic databases Medline, Embase (Ovid) and Scopus up to 13 July 2021. The following terms, including synonyms and closely related words, were used as index terms and free-text words: "differentiated or HPV-independent" and "vulvar intraepithelial neoplasia" or "dVIN" (Table S1). No limits were applied, and duplicate articles were excluded using EndNote.

Eligibility Criteria and Study Selection

Five medical doctors and experts in vulvar pathology (MCGB, MvB, RFMV, NBT, FOV) independently screened all titles and abstracts for eligibility according to predetermined in- and exclusion criteria, using Rayyan QCRI. This was followed by independent full-tekst screening of the selected articles. Articles were regarded as eligible to be included whenever one or both of the following aspects were assessed: VSCC risk in patients with dVIN and/or the risk of recurrent VSCC when arising in a background of dVIN. Case reports and articles of which full text was unavailable were excluded. Studies were also excluded if no separate analyses had been performed for high-grade VIN lesions to discriminate between vHSIL and dVIN. Reference lists of included articles were cross-checked manually to identify any additional studies. Selected articles were discussed in a consensus meeting with all reviewers to reach a final selection.

Data Extraction and Analysis

Data from selected studies were extracted from the full text or tables by four reviewers (MCGB, RFMV, NBT, FOV). Data extraction included first author, year of publication, country of investigation, study design, type of cohort, inclusion period and criteria, number and type of cases included (dVIN without history of VSCC and/or dVIN adjacent to VSCC), age at dVIN and/or dVIN adjacent to VSCC diagnosis, follow-up time, number of primary and/or recurrent VSCC cases and time to progression to (recurrent) VSCC. The absolute risk of primary and/or recurrent VSCC development was extracted or calculated if adequate data were provided. Absolute risk was calculated by dividing the number of cases developing primary and/or recurrent VSCC

by the number of dVIN and/or dVIN adjacent to VSCC cases. Data were analyzed in a descriptive manner if meta-analysis could not be performed. The data were tabulated in Microsoft Excel version 2016 (Microsoft Corporation, Redmond,WA, USA).

Critical Appraisal

The quality and risk of bias of included studies were independently assessed by two reviewers (NBT, FOV) using the Quality in Prognostic Studies (QUIPS) tool of the Cochrane Prognosis Methods Group.(16) The QUIPS tool includes the following six bias domains: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical analysis and reporting. Studies were evaluated for their reliability and eligibility for each of the six domains, rating as low, moderate or high potential risk of bias. Discrepancies were resolved in a consensus meeting.

Results

Search Results

The systematic literature search and selection process is outlined in the PRISMA flow diagram (Figure 1).(15) A total of 901 articles were found in Medline, Embase and Scopus databases using our selected search. After removal of duplicates, 455 articles were selected for primary screening, after which 418 articles were excluded based on title and abstract. Of the 37 articles sought for retrieval, full-text reports were not available of 7 articles. After full-text assessment, an additional 23 articles were excluded which did not meet our inclusion criteria. This selection procedure resulted in seven articles to be included for analysis.

Study Characteristics

The characteristics of all seven included studies are summarized in Table 1. Three studies were carried out in the Netherlands by three different institutes (7, 17, 18), of which two were population-based studies and one was a center-based study. The other four studies were center-based studies carried out in Austria (19), Canada (20), the UK (21) and the USA (22). All had a retrospective cohort study design. The number of included dVIN cases varied from 7 to 197. The study periods in which dVIN cases were included varied from 5 to 20 years, with cases included between 1985 and 2016. From one study, data on the inclusion period was not available.

Table 1. Characteristics of included studies

Study (Year)	Country	Study Design	Type of Cohort
Yang et al. (2000) [22]	USA	Retrospective cohort study	Center-based
Van de Nieuwenhof et al. (2009) [18]	The Netherlands	Retrospective cohort study	Population-based
Regauer et al. (2016) [19]	Austria	Retrospective cohort study	Center-based
Thuijs et al. (2020) [7]	The Netherlands	Retrospective cohort study	Population-based
McAlpine et al. (2017) [20]	Canada	Retrospective cohort study	Center-based
Eva et al. (2008) [21]	UK	Retrospective cohort study	Center-based
Te Grootenhuis et al. (2019) [17]	The Netherlands	Retrospective cohort study	Center-based

[1] Referred to as simplex VIN in the article. Abbreviations: dVIN, differentiated vulvar intraepithelial neoplasia (cases without history of vulvar cancer); dVIN/VSCC, dVIN adjacent to vulvar squamous cell carcinoma; HPV, human papillomavirus; LS, lichen sclerosus; NA, not available; NOS, not otherwise specified; UK, United Kingdom; USA, United States of America; VIN, vulvar intraepithelial neoplasia.

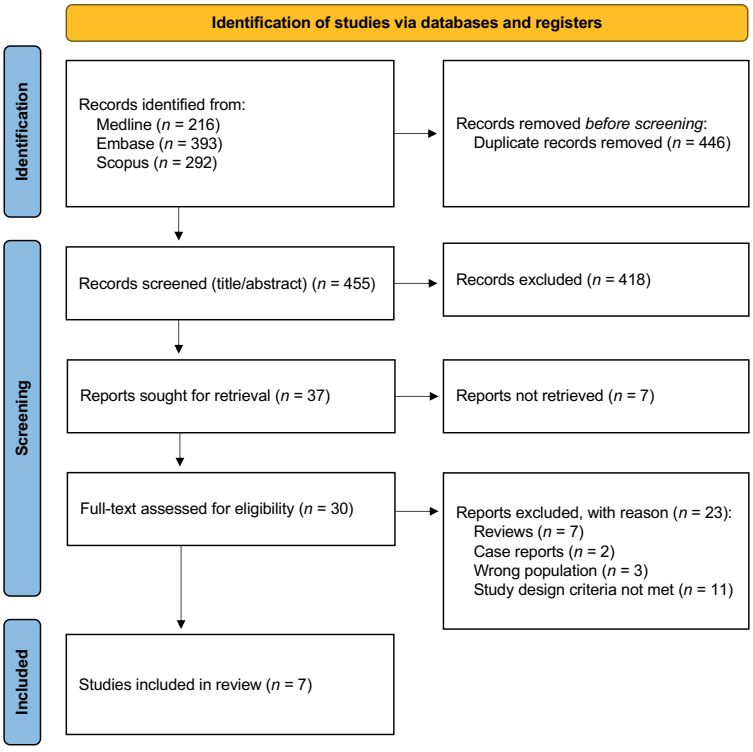

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for selection of studies.(15)

Inclusion Period	Number and Type of Cases	Inclusion Criteria dVIN
-	8 dVIN[1]	Revision of pathology slides
1992-2005	67 dVIN	Pathology reports describing dVIN as differentiated VIN, VIN simplex type, VIN NOS with LS and/or a high-risk HPV-negative result
2004-2016	16 dVIN	Revision of pathology slides
1991-2011	12 dVIN	Pathology reports describing dVIN as differentiated VIN, vulvar dystrophy with atypia or simplex VIN
1985-2005	7 dVIN and 18 dVIN/VSCC	Revision of pathology slides
2000-2005	44 dVIN/VSCC	Pathology reports describing dVIN adjacent to VSCC
2000-2010	197 dVIN/VSCC	Revision of pathology slides

Risk of Bias of All Included Studies

Selected studies underwent quality assessment according to the QUIPS tool (Table 2). The more recent published studies had an overall lower risk of potential bias for all domains. All of the studies scored a moderate to high potential risk of bias concerning the study confounding domain. This was the most common methodological weakness, as important confounders relevant to primary and recurrent VSCC risk, such as treatment of primary dVIN or pathologic free margins in dVIN associated VSCC, were often not taken into account or measured inadequately.

Outcome of Objectives

Primary VSCC Risk in dVIN

Five studies, including two population-based and three center-based studies, studied the risk of developing primary VSCC in dVIN patients (Table 3).(7, 18-20, 22) Sample sizes varied from 7 to 67 dVIN patients, with age ranging from 67 to 75 years. The absolute cancer risk in women with dVIN was calculated with the total number of VSCC cases in each separate dVIN cohort and ranged between 33 and 86% across the five included studies.

The time to primary VSCC progression was investigated in four of the five studies. (7, 18, 20, 22) The median interval between dVIN diagnosis and VSCC diagnosis ranged from 9 to 23 months. Pooling of results was not possible because two studies did not report any information regarding follow-up time (18, 19) and the other three studies reported differences in follow up time, ranging from 72 to 167 months. (7, 20, 22) No correlation was found between the duration of follow-up and the cancer risk observed.

Recurrent VSCC Risk in dVIN

Three center-based studies investigated the risk of recurrent vulvar cancer in patients treated for dVIN associated VSCC (Table 3).(17, 20, 21) Sample sizes varied from 18 to 197 patients with dVIN adjacent to VSCC, with age ranging from 73 to 76 years. Overall, the recurrence risk ranged between 32 and 94%. The median time to recurrence was reported in the two studies with the highest VSCC recurrence risk and was 13 and 32 months.(17, 20)

Higher local VSCC recurrence rates were observed when dVIN was specifically located in the resection margins compared to patients with dVIN found adjacent to the tumor but not in the resection margins (61% versus 42% after 10 years, respectively, $p = 0.002$).(17) The study which reported the highest recurrent cancer risk (94%)

Table 2. Risk of bias summary: judgement of each domain for all included studies using the Quality of Prognostic Studies (QUIPS) tool [16].

	Study Participation	Study Attrition	Prognostic Factor Measurement	Outcome Measurement	Study Confounding	Statistical Analysis and Reporting
Yang et al. (2000) [22]	High	High	Moderate	Low	Moderate	Moderate
Van de Nieuwenhof et al. (2009) [18]	Low	Low	Moderate	Low	High	Low
Regauer et al. (2016) [19]	High	High	Moderate	High	High	High
Thuijs et al. (2020) [7]	Low	Low	Moderate	Low	High	Low
McAlpine et al. (2017) [20]	Low	Moderate	Low	Low	Moderate	Low
Eva et al. (2008) [21]	Moderate	High	Moderate	High	High	Moderate
Te Grootenhuis et al. (2019) [17]	Low	Moderate	Low	Low	High	Low

reported that 7/20 (35%) and 15/20 (75%) surgical specimens had positive resection margins for invasive carcinoma or dVIN, respectively.(20) All patients with positive margins for VSCC received adjuvant radiotherapy in this study.

Table 3. Outcome of primary objectives for all included studies

Study (year)	Number and Type of Cases	Age	Follow-Up Time
		Years, Median (Range)	Months, Median (Range)
Yang et al. (2000) [22]	8 dVIN[1]	67.5 (55-82)	Mean 85.5 (14-169)
Van de Nieuwenhof et al. (2009) [18]	67 dVIN	67	-
Regauer et al. (2016) [19]	16 dVIN	-	-
Thuijs et al. (2020) [7]	12 dVIN	70.3 (40-85)	167 (4-329)[2]
McAlpine et al. (2017) [20]	7 dVIN	Mean 75.1[3]	72
	18 dVIN/VSCC	Mean 75.8[3]	-
Eva et al. (2008) [21]	44 dVIN/VSCC	-	-
Te Grootenhuis et al. (2019) [17]	197 dVIN/VSCC	73 (26-100)[4]	80 (0-204)

[1] Referred to as simplex VIN in the article;
[2] Follow-up time for entire study population (n = 894);
[3] Mean age;
[4] Median age for entire study population (n = 287);
[5] Number of cases calculated from reported 10-year recurrent risk in dVIN/VSCC cohort;
[6] Time to progression for entire study population (n = 287), did not differ significantly between adjacent precursor lesion groups (p = 0.08).
Abbreviations: dVIN, differentiated vulvar intraepithelial neoplasia (cases without history of vulvar cancer); dVIN/VSCC, dVIN adjacent to vulvar squamous cell carcinoma; NA, not available; VSCC, vulvar squamous cell carcinoma.

Primary VSCC Risk in dVIN			Recurrent VSCC Risk in dVIN/VSCC		
Risk of Primary VSCC		Time to Progression	Risk of recurrent VSCC		Time to progression
Number of Primary VSCC	Absolute Risk (%)	Months, Median (Range)	Number of Recurrent VSCC	Absolute Risk (%)	Months, Median (Range)
3	37.5	9 (6-55)	-	-	-
22	32,8	22.8 (3-84)	-	-	-
9	56.3	-	-	-	-
7	58.3	16.8	-	-	-
6	85.7	22.8	-	-	-
-	-	-	17	94.4	13.2
-	-	-	14	31.8	-
-	-	-	94[5]	47,7	32 (0-202)[6]

Discussion

This is the first systematic review summarizing the primary and recurrent risk of VSCC in women with dVIN, including the time to primary and recurrent VSCC. Patients with dVIN have a high risk of developing VSCC, with a reported absolute primary VSCC risk of 33–86% (7, 18-20, 22) and a recurrent VSCC risk of 32–94%.(17, 20, 21) The median time to primary vulvar cancer progression varies widely, but nearly all cases occurred within 2 years after dVIN diagnosis.(7, 18, 20, 22) VSCC recurrences after primary VSCC with adjacent dVIN occurred mostly within 3 years after primary vulvar cancer diagnosis.(17, 20)

There are a number of limitations of this review. First of all, only seven studies met our inclusion criteria with high heterogeneity between the studies. In contrary to the broad consensus that dVIN is a condition with a high cancer risk, the low number of studies on this subject is illustrative of the relatively limited amount of research that has been done on dVIN. Moreover, these studies are difficult to conduct as solitary dVIN is a rare diagnosis. Due to rapid progression to carcinoma, dVIN is often diagnosed adjacent to VSCC, explaining the higher sample sizes observed in studies on recurrent cancer risk compared to those studying the primary cancer risk.

A second limitation is the selection bias observed in a number of studies. Interestingly, the study with the largest cohort reported the lowest absolute primary vulvar cancer risk, which is a population-based study,(18) whereas the highest primary cancer risk was found in the study with the smallest cohort, which is a center-based study.(20) Nearly all centerbased studies on solitary dVIN have relatively small sample sizes. Although center-based cohorts are more prone to selection bias, most of these studies did revise the pathology slides to confirm dVIN diagnosis, leading to a less biased selection.(17, 19, 20, 22)

In contrast, the two population-based studies contain more cases of dVIN and lack selection bias.(7, 18) Both studies observed that dVIN occurs more frequently in older women, with a peak incidence amongst women between age 75 and 85 years. Although these are more representative cohorts of the general population, histopathologic review of cases was not performed. Moreover, the two studies used different definitions of dVIN when selecting cases. In addition to 'differentiated VIN' and 'VIN simplex type', Van de Nieuwenhof et al. also included cases with 'VIN NOS (not otherwise specified) with LS and/or an HPV negative result' in the dVIN group, while Thuijs et al. did not include these latter cases. This seems to influence not only the number of included dVIN cases (67 vs. 12, respectively), but also the

observed primary cancer risk (33% vs. 58%, respectively). This is further supported by a subgroup analysis performed by Thuijs et al., showing a 10-year cumulative VSCC incidence of 38% in 43 patients who had both vHSIL and LS, which is comparable to the cancer risk in the dVIN population reported by van de Nieuwenhof et al.(7, 18)

Another limitation of this review is the retrospective design of all studies. This most likely resulted in the lack of information on relevant confounders, such as age, treatment, comorbidities and medical history. An important confounder which was rarely reported is the effect of dVIN treatment on the primary cancer risk. As spontaneous regression of dVIN is unlikely to occur, patients with dVIN are surgically treated by wide local excision with the aim of free resection margins.(23) Needless to say, dVIN patients treated by radical excision will probably have a lower risk of subsequent malignancy. Only one study reported on surgical treatment in patients with dVIN without a history of VSCC.(22) In this study, the three patients who developed VSCC only had a diagnostic biopsy taken, whereas the other patients who did not develop VSCC underwent total or partial vulvectomy or an excision of the dVIN lesion. This emphasizes the importance of radical excision of dVIN to prevent malignant progression.

Nevertheless, even after treatment, recurrent dVIN lesions are common as the remaining anogenital area adjacent to the removed dVIN lesion is often abnormal or affected with LS. Hence, close and lifelong follow-up as well as adequate treatment of both LS and dVIN is needed. The primary treatment of LS consists of maintenance therapy with ultra-potent topical corticosteroids (UTCS), which has shown to prevent the progression towards VSCC.(24) As dVIN mostly arises in a background of LS, UTCS maintenance treatment after surgical excision of dVIN should be considered to prevent recurrent dVIN and subsequent VSCC.

Finally, the studies on recurrent VSCC risk reported a wide variance in the resection margin status of the surgical specimens. In the study with the highest vulvar cancer recurrence rate amongst patients treated for dVIN associated VSCC, 35% of primary surgical specimens had positive margins for invasive carcinoma.(20) This inherently influences the chance for recurrent vulvar cancer, which therefore must be taken into account when interpreting this result. This also applies to resection margins being positive for dVIN. Although only one study investigated this, the presence of dVIN in the resection margin leads to a higher risk of recurrent cancer compared to patients without positive margins for dVIN (10-year local recurrence risk of 61% vs. 42%, respectively).(17) This implies that not only the vulvar cancer should be radically excised, but also all the adjacent dVIN to prevent recurrent disease. Current

guidelines do not give clear recommendations on what to do when dVIN is present in the pathologic resection margin after VSCC excision.(25-27) Consequently, current clinical practice with regards to positive dVIN margins depends on the expert opinion of the treating gynecologic oncologist. However, given the increased recurrent VSCC risk in patients with positive resection margins for dVIN, it is reasonable to consider re-excision of surgical scars in such cases.

Diagnosing dVIN is challenging for clinicians, both histologically and clinically. The histopathologic morphology may vary considerably. In recent years, two new HPVindependent precursors of VSCC have been identified, termed differentiated exophytic vulvar intraepithelial lesion (DE-VIL) and vulvar acanthosis with altered differentiation (VAAD).(28-30) These entities show overlapping morphology with dVIN, and previous studies suggest that a molecular association exists with verrucous carcinoma, a rare variant of HPV-independent VSCC.(29, 31) However, many of the histological features, such as parakeratosis, absence of a granular layer, premature keratinization and nuclear atypia, are not specific for dVIN, DE-VIL and VAAD only.(32-34) HPV-independent precursors of VSCC are therefore easily mistaken for inflammatory atypia, squamous hyperplasia or other non-neoplastic epithelial disorders. A study in 2011 found that 42% of LS cases were reclassified as dVIN lesions after revision by a specialized gynecologic pathologist.(35) Yet, even for experienced pathologists with gynecological expertise the diagnosis remains challenging. A study evaluating the reproducibility of the histopathologic diagnosis of dVIN uncovered a low interobserver agreement between pathologists, which did improve in gynecologic pathologists after specific guidelines with histological characteristics were provided.(36) More recently, a survey amongst pathologists uncovered that basal layer atypia was the only essential feature of dVIN over which consensus was reached.(34) Thus, the histopathological diagnosis of dVIN and other HPV-independent precursors of VSCC can be subtle and prone to misdiagnosis.

Likewise, clinical recognition of dVIN is difficult due to its varying presentation. It often presents as a focal grey-white or red colored roughened surface, but may also appear as a thickened white plaque, wart-like or as an atrophic or ulcerative lesion.(10, 37) After surgical treatment of VSCC, it becomes even more difficult to clinically visualize a dVIN lesion. Surgical scarring can mimic LS and vulvar neoplasia, resulting in a possible residue or recurrent dVIN to be easily mistaken for fibrotic tissue. Therefore, lack of recognition and misdiagnosis are likely contributing factors leading to underdiagnosis and –treatment of dVIN patients and subsequent progression towards VSCC.

Conclusions

The results of this review confirm the clinical impression that the risk of developing primary and recurrent VSCC in dVIN is high with a short time to cancer progression. Adequate treatment by radical excision and careful surveillance of patients with dVIN, preferably in a gynecologic oncology center with multidisciplinary expertise, is therefore highly recommended. Patients ought to be well informed on the high cancer risk and the need for frequent visits with regular biopsies, especially in the presence of symptoms. Rapid progression to VSCC and lack of recognition cause dVIN to be a rare finding. Novel techniques and additional biomarkers are currently being investigated to aid in diagnostics of this rare and aggressive disease. Prospective studies with larger well-defined cohorts, preferably population-based, are highly needed to further investigate the cancer risk in dVIN.

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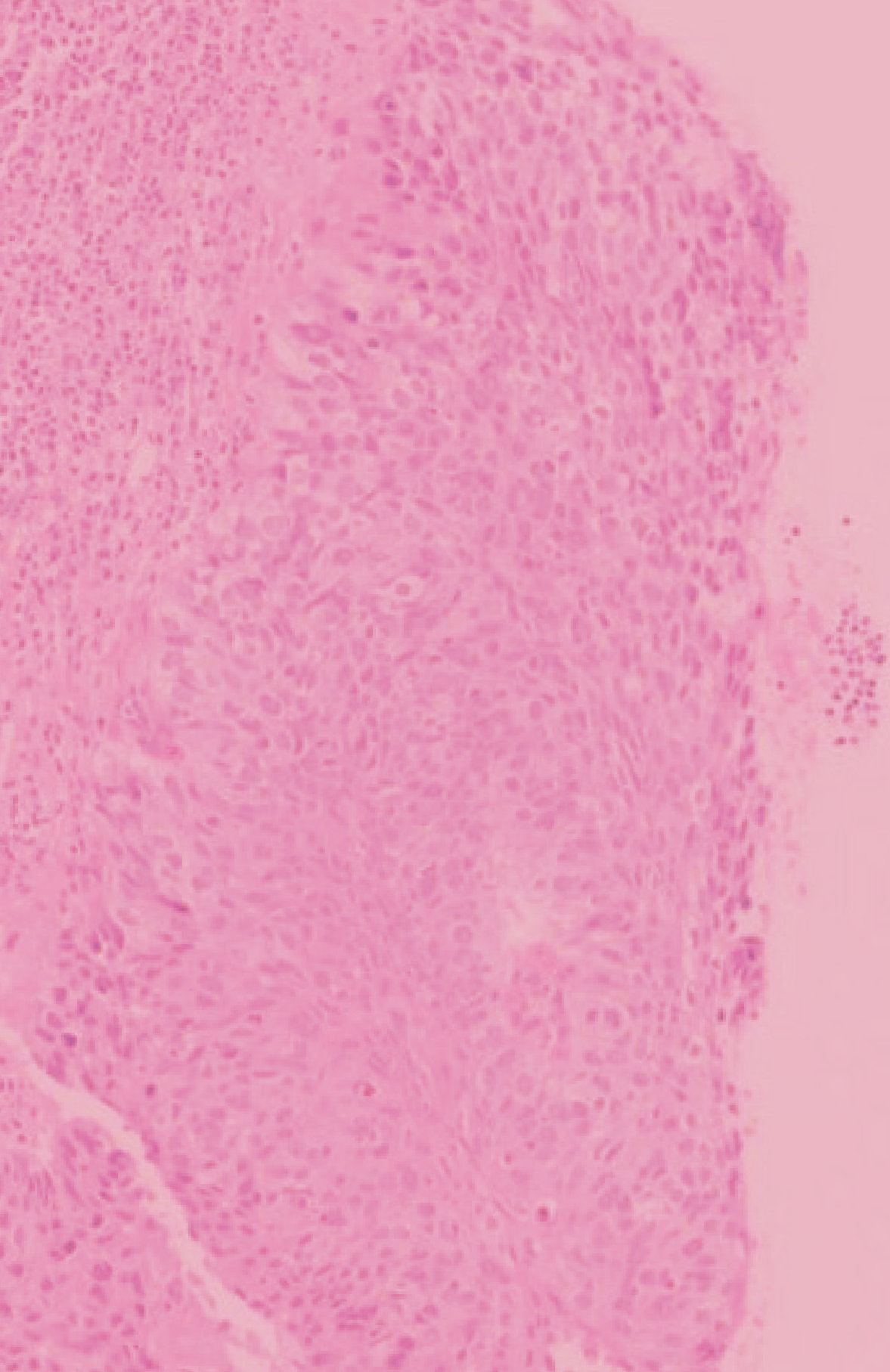

CHAPTER 4

DNA methylation markers for cancer risk prediction of vulvar intraepithelial neoplasia

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Abstract

Current clinical and histological classifications are unable to determine the risk of vulvar squamous cell carcinoma (VSCC) in high-grade vulvar intraepithelial neoplasia (VIN), making prognostic biomarkers highly needed. We studied host-cell DNA methylation markers in HSIL and dVIN without VSCC, in HSIL and dVIN adjacent to VSCC, and in human papillomavirus (HPV) positive and negative VSCC, relative to control vulvar tissues. A series of 192 formalin-fixed paraffin-embedded vulvar samples, including VSCC (n=58), VIN adjacent to VSCC (n=30), VIN without VSCC during follow-up (n=41) and normal vulvar tissues (n=63), were tested for 12 DNA methylation markers with quantitative multiplex methylation-specific PCR (qMSP). HPV status was determined by $p16^{INK4A}$ immunohistochemistry and high-risk HPV PCR analysis. Logistic regression analyses were used to determine methylation patterns and methylation marker performance for VIN and VSCC detection. Methylation markers showed significantly higher methylation levels with increasing severity of disease. VIN adjacent to VSCC showed a similar methylation-high pattern as VSCC, while VIN without VSCC displayed a heterogeneous methylation pattern. Vulvar carcinogenesis is associated with increased DNA methylation. Higher DNA methylation levels in VIN seem to reflect higher cancer risk, emphasizing the high potential of DNA methylation biomarkers in the diagnostic workup of VIN. As a next step, longitudinal studies are needed to verify the prognostic value of methylation biomarkers as a clinical tool for stratification of cancer risk in women with VIN.

Introduction

Vulvar squamous cell carcinoma (VSCC) accounts for approximately 5% of gynecological malignancies and 95% of all vulvar malignancies. The precursor lesion of VSCC is high-grade vulvar intraepithelial neoplasia (VIN). VIN is classified into high-grade squamous intraepithelial lesion (HSIL), which is human papillomavirus (HPV) related, and differentiated VIN (dVIN), which is independent of HPV and associated with lichen sclerosus (LS).[1-3] HSIL, previously known as usual type of VIN (uVIN), is the most common type of VIN, occurring mainly in women aged between 35 and 50 years. Treatment modalities range from topical imiquimod to surgery, leading to somatic and psychosexual morbidity.[4] Despite the relatively low absolute cancer risk of HSIL, i.e., 2.3-6.6% after 3 years, all HSIL are treated to prevent cancer.[5-7] Current clinicopathological parameters are insufficient to accurately predict individual cancer risk. To reduce overtreatment and associated morbidity, biomarkers that could predict individual cancer risk in women with HSIL are urgently needed.

The molecular events leading to the development of VSCC through VIN are not yet well understood. Few studies have examined DNA mutation or copy number alterations and correlated these with the risk of progression in VIN, but no prognostic biomarkers ready for clinical use have been found so far.[8, 9] Epigenetic changes, such as hypermethylation of promoter cytosine-phosphate-guanine (CpG) islands of tumor suppressor genes, can contribute to the development of cancer by gene silencing.[10] In HPV-related cervical and anal disease, DNA methylation testing has provided promising biomarkers for the identification of precursors with a presumed high cancer risk.[10-13] Various methylation markers associated with HPV-induced anogenital carcinogenesis have been discovered, including *ASCL1, CADM1, FAM19A4, GHSR, LHX8, MAL, miR124-2, PHACTR3, PRDM14, SST, ZIC1* and *ZNF582*.[12, 14, 15] In vulvar (pre)malignancies, few data exist on DNA methylation of host cell genes.

In this study, we tested above 12 methylation markers in a large and well-defined series of HPV positive and negative vulvar carcinomas and VIN, divided into VIN without progression to VSCC during long-term follow-up and VIN adjacent to VSCC, to assess the potential value for cancer risk prediction of VIN.

Materials and methods

Patients and samples

This study included 192 vulvar samples from 192 women, categorized into 4 groups: normal (control) vulvar tissues (n = 63), VIN without VSCC (n = 41), VIN adjacent to VSCC (n = 30) and VSCC (n = 58). VIN without VSCC refers to VIN lesions detected in women that did not develop VSCC during a median follow-up time of 17.8 years (range 1.0 to 27.1 years). To confirm absence of VSCC, follow-up data with nationwide coverage, were retrieved from PALGA, the nationwide network and registry of histopathology and cytopathology in the Netherlands.[16] The group of VIN adjacent to VSCC was used as surrogate for the most advanced stage of VIN, representing VIN with a high progression risk to cancer. Both HSIL, dVIN and VSCC tissues were retrieved from the pathology archives of Amsterdam UMC and Antoni van Leeuwenhoek hospital, in Amsterdam, the Netherlands, between 1984 and 2015. Compared to regular care, VIN adjacent to VSCC and VSCC were enriched for HPV-positive cases.[9] The control group comprised vulvar samples from healthy patients collected during aesthetic genital procedures in the "V Klinieken" in Leiden, the Netherlands, or during reconstructive genital procedures in Amsterdam UMC, location VUmc, in 2018 and 2019.

Histopathology

Formalin-fixed, paraffin embedded (FFPE) tissue blocks were sectioned using the sandwich method. The first and last sections (3 µm) were used for hematoxylin–eosin staining to ensure the presence of the same lesion, and in-between sections (10 µm) were collected in sterile PCR tubes for DNA isolation. Precautions were taken to avoid cross-contamination as described before.[17]

VIN adjacent to VSCC samples were selected in women with VSCC with sufficient adjacent VIN. VIN adjacent to VSCC and VSCC were harvested by laser-capture microdissection when present in one tissue block. For the selection of tissues, all slides were reviewed by a gynecopathologist (M.C.G.B.) and a senior resident in pathology (N.B.T.). Histological subtypes of VIN (HSIL or dVIN) and VSCC (keratinizing or basaloid/warty), as well as the International Federation of Gynecology and Obstetrics (FIGO) stage of all VSCC cases was documented.

DNA isolation

DNA was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was eluted with the easyMAG 3 elution buffer (bioMérieux, Boxtel, the Netherlands). DNA concentration was measured using Qubit (Thermo Fisher Scientific Inc, Qiagen).

DNA methylation analysis using multiplex quantitative methylation-specific PCR (qMSP)

DNA was bisulfite-converted using the EZ-DNA Methylation kit (Zymo Research, Orange, CA, USA).[18] For methylation analysis, EpiTect MethyLight Master Mix (Qiagen) was used, together with fluorescent dye-labelled probes, 50 ng of bisulfite-converted DNA and 100-300 nM of each primer.[19]

We analyzed 12 DNA methylation markers in 4 multiplex qMSP assays, each assay targeting 3 markers and the reference gene, β-actin (*ACTB*).

Multiplex qMSPs targeting *GHSR/SST/ZIC1* and *ASCL1/LHX8/ZNF582* were performed on the ViiA7 Real-Time PCR System with inclusion of a calibrator (Applied Biosystems, Foster City, CA, USA) and multiplex qMSPs targeting *FAM19A4/PHACTR3/PRDM14* and *CADM1/MAL/miR124-2* were run on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems).[18-20] All samples were first tested for the 6 markers *ASCL1, LHX8, ZNF582, GHSR, SST* and *ZIC1*. Due to limited availability of DNA, multiplex qMSP *CADM1/MAL/miR124-2* was tested on 129/192 samples and multiplex qMSP *FAM19A4/PHACTR3/PRDM14* on 143/192 samples. A Ct ≤32 for *ACTB* indicated sufficient DNA and adequate bisulfite conversion.[20] Invalid test results (i.e. *ACTB* Ct >32*)* were obtained from 3/129 samples tested for qMSP *CADM1/MAL/miR124-2*. No invalid results were obtained from the remaining three multiplexes.

ΔCt or ΔΔCt ratios were computed using the comparative Ct method, normalizing target Ct values to respectively *ACTB* or to *ACTB* and a calibrator.[21]

HPV status

Immunostaining of $p16^{INK4a}$ was performed with mouse monoclonal antibodies against the $p16^{INK4a}$ antigen (clone E6H4; Roche, Basel, Switzerland), using the Optiview detection kit with the automated BenchMark ULTRA IHC/ISH system (Roche). $p16^{INK4A}$ immunohistochemistry was scored positive when diffuse or block staining was observed and negative with a negative or patchy staining pattern.[22]

High-risk HPV DNA-testing was performed using the QIAscreen® HPV PCR Test (QIAgen, Hilden, Germany), as described previously for use on FFPE biopsy specimens.[23] The assay is directed against the E7 gene of 15 (probably) high-risk HPV genotypes, i.e. 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68, with partial genotype information (HPV16 and -18).[24] Beta-globin served as internal quality control. Samples were considered invalid for PCR testing when the cycle threshold (Ct) >30 for beta-globin and no HPV was found.

HPV status was determined in all VIN and VSCC and not in controls. HPV status was considered positive when $p16^{INK4A}$ and/or HPV PCR were positive, and negative when $p16^{INK4A}$ was negative and HPV PCR was negative or invalid.

Statistical analysis

To evaluate methylation levels per disease category, boxplots were computed from the log2-transformed Δ(Δ)Ct ratios of the markers. Differences in methylation levels between disease categories were assessed using the Kruskal-Wallis test, followed by post hoc testing using the Mann-Whitney U test and by Bonferroni multiple testing correction in cases with significant results.

Univariable logistic regression analyses were performed on log2-transformed Δ(Δ)Ct ratios of 6/12 markers with complete methylation data (*ASCL1, LHX8, ZNF582, GHSR, SST* and *ZIC1*). A logistic regression model built for normal versus VSCC was used to visualize methylation patterns by calculating predicted probabilities of underlying VSCC for each sample and marker, with values ranging from 0 to 1. To assess the potential diagnostic value of the 6 methylation markers for the clinical management of women with VIN we compared VIN without VSCC versus controls and VIN without VSCC versus VSCC by visualizing receiver operating characteristic (ROC) curves, assessed through the area under the curve (AUC).

Logistic regression analysis was performed in R open source software version 4.0.2 and the pROC package was implemented for ROC analysis. All other statistical analyses were performed in IBM SPSS Statistics software for Windows version 24.0 (IBM Corporation, Armonk, NY). Reported p values were 2-sided. $P<0.05$ was considered statistically significant and was scored as marginal evidence ($0.01<p0.05$), moderate evidence ($0.001<p<0.01$) and strong evidence ($p<0.001$).

Results

Baseline characteristics

Baseline characteristics and HPV status per disease category of the study population are shown in Table 1. Median age was highest for patients with VSCC (72.5 years, range 36-95) and lowest for controls (28.0 years, range 18-57). FIGO stages of the VSCCs were stage Ia in 4, Ib in 33, IIIa in 10, IIIb in 2 and IIIc in 9 tumors.

HPV status was positive in 90.2% (37/41) of VIN without VSCC, in 60.0% (18/30) of VIN adjacent to VSCC and in 46.6% (27/58) of VSCC. All HPV-positive VIN had HSIL

morphology and all HPV-negative VIN had dVIN morphology. The keratinizing and the basaloid/warty subtype of VSCC was found in 59.3% and 40.7% of the HPV-positive VSCCs and in 83.9% and 16.1% of the HPV-negative VSCCs, respectively. Predominant HPV genotype was HPV16, accounting for respectively 80.6% and 100% of all HPV-positive VIN without VSCC and VSCC. Multiple infections were found in 3.8% (3/80) of HPV PCR-positive samples.

Table 1. Baseline characteristics and HPV status per disease category

		Control	VIN		VSCC
			without VSCC	adjacent to VSCC	
Number		63	41	30	58
Median age (range)		28.0 (18-57)	4.0 (21-86)	66.0 (36-92)	72.5 (36-95)
Histological subtype of VIN (%)	HSIL		37 (90.2)	18 (60.0)	
	dVIN		4 (9.8)	12 (40.0)	
Histological subtype of VSCC (%)	Keratinizing				41 (70.7)
	Basaloid/warty				17 (29.3)
HPV status (%)	Positive hr-HPV		37 (90.2)	18 (60.0)	27 (46.6)
	HPV16		29 (70.7)	12 (46.7)	26 (44.8)
	HPV18		0 (0.0)	0 (0.0)	0 (0.0)
	Non-16/18		7 (17.1)	4 (20.0)	0 (0.0)
	HPV16 & non-16/18		1 (2.4)	2 (6.7)	0 (0.0)
	Not determined		0 (0.0)	0 (0.0)	1 (1.7)
	Negative		4 (9.8)	12 (40.0)	31 (53.4)

Abbreviations: HSIL = high-risk squamous intraepithelial lesion, dVIN = differentiated VIN, HPV = human papillomavirus, VIN = high-grade vulvar intraepithelial neoplasia, VSCC = vulvar squamous cell carcinoma.

DNA Methylation levels in different vulvar disease categories

Methylation levels of 11/12 markers (except for CADM1) increased significantly with severity of disease. Significantly higher methylation levels were found for all markers in VSCC compared to controls, for 11/12 markers in VIN without VSCC compared to controls, for 10/12 markers in VIN without VSCC compared to VSCC, and for 8/12 markers in VIN without VSCC compared to VIN adjacent to VSCC (Figure 1). None of the markers showed a significant difference between VIN adjacent to VSCC and VSCC.

DNA Methylation levels in relation to HPV status

In HPV-positive samples, 10/12 markers (except for CADM1 and MAL) showed significantly higher methylation levels with increasing severity of disease (Supplementary Figure 1). For CADM1 and MAL, a trend towards higher methylation levels with increasing severity of disease was seen, but significance was not reached, likely because of small sample sizes.

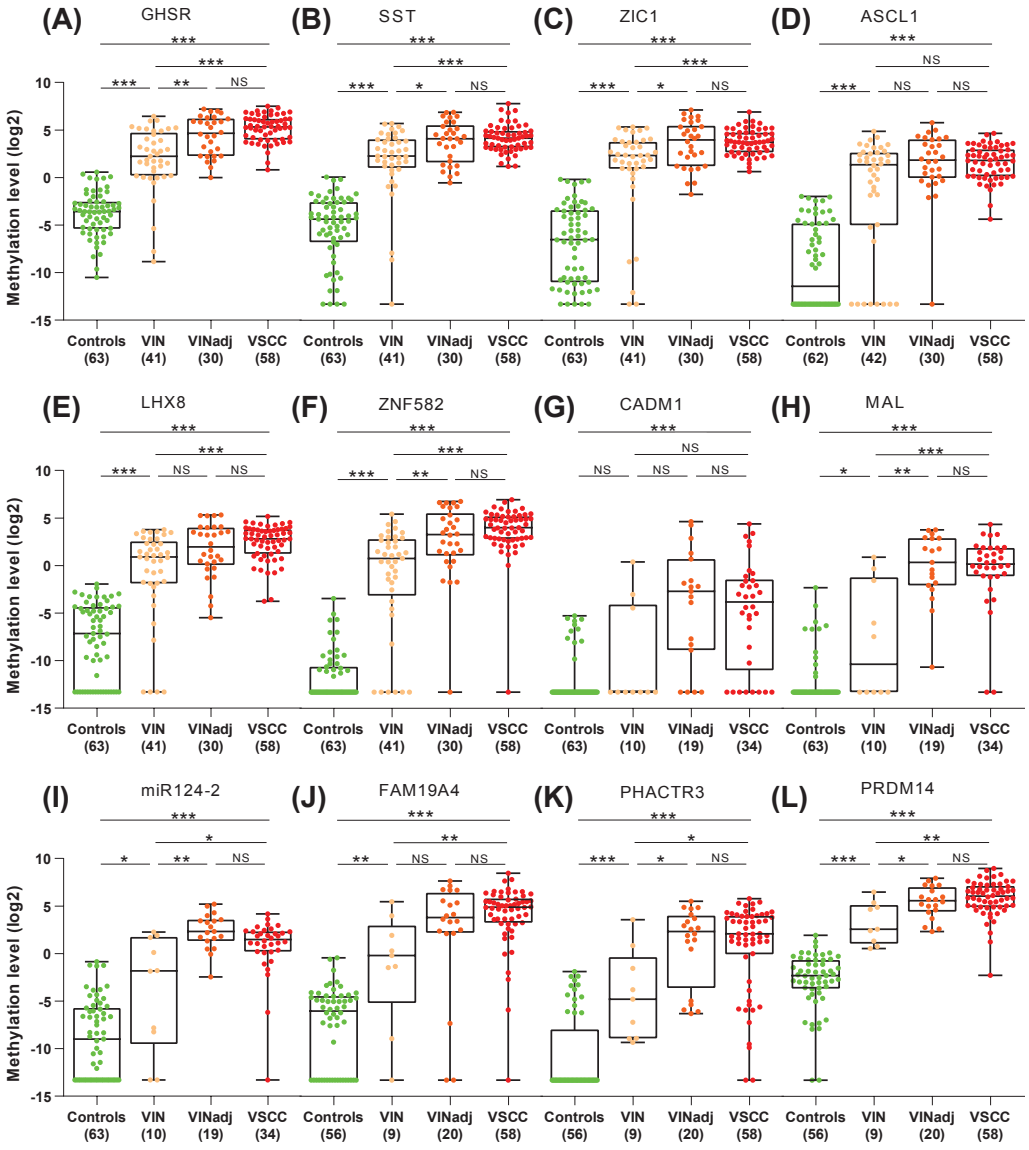

Figure 1. DNA methylation levels shown relative to the reference gene ACTB (log2-transformed Δ(Δ)Ct ratios; y-axis) for the 4 disease categories (x-axis) for 12 markers: A, GHSR; B, SST; C, ZIC1; D, ASCL1; E, LHX8; F, ZNF582; G, CADM1; H, MAL; I, miR124-2; J, FAM19A4; K, PHACTR3; and L, PRDM14. Differences between histological categories upon Kruskal-Wallis test, followed by post hoc testing using the Mann-Whitney U test and Bonferroni multiple testing correction: *P < .05 (marginal evidence), **P < .01 (moderate evidence), ***P < .001 (strong evidence), NS, not significant. VIN, high-grade vulvar intraepithelial neoplasia; VINadj, VIN adjacent to VSCC; VSCC, vulvar squamous cell carcinoma.

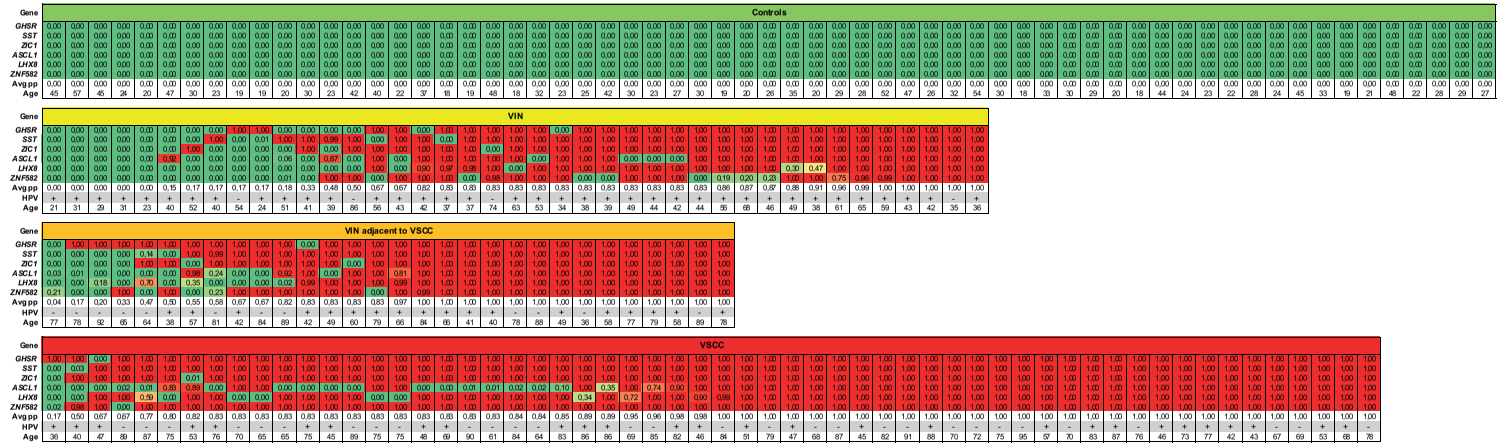

Figure 2. DNA methylation pattern of six methylation markers (*GHSR, SST, ZIC1, ASCL1, LHX8* and *ZNF582*) across all four histological disease categories. Predicted probabilities (pp) per sample (column) are colored from green (pp of 0, ie, low) to red (pp of 1, ie, high). In each disease category, samples are ordered based on their average pp (Avg pp). HPV status of the samples and age of the patients is displayed at the bottom of each disease category. +, HPV positive; –, HPV negative; VIN, high-grade vulvar intraepithelial neoplasia; VSCC, vulvar squamous cell carcinoma

In HPV-negative samples, all markers showed significantly higher methylation levels with increasing severity of disease (Supplementary Figure 2). However, dVIN without VSCC was not tested for 6 markers (*CADM1, MAL, miR124-2, FAM19A4, PHACTR3 and PRDM14)*, due to limited DNA availability.

Methylation patterns and diagnostic performance of individual methylation markers

The DNA methylation patterns, depicted by predicted probabilities of underlying VSCC for each sample separately, are shown in Figure 2. Controls uniformly showed very low predicted probabilities, consistent with a methylation-low pattern. VSCCs showed uniformly high predicted probabilities, consistent with a methylation-high pattern, with the lowest average predicted probability of the six markers equal to 0.17. Predicted probabilities were also consistently high across markers, with the exception of ASCL1, showing relatively low predicted probabilities in VSCC. VIN adjacent to VSCC showed predominantly high average predicted probabilities, similar to VSCC. VIN without VSCC demonstrated a heterogeneous methylation pattern, with samples displaying both low and high individual predicted probabilities (respectively green and red boxes in Figure 2).

Within individual disease categories, age and HPV were equally represented across low and high average predicted probabilities, with the exception of VIN adjacent to VSCC, in which the lowest 5 average predicted probabilities were found in dVIN. Marker-specific ROC curves demonstrated AUCs of 0.829 to 0.931 when discriminating between VIN without VSCC and controls (Figure 3A), and AUCs of 0.601 to 0.855 when discriminating between VIN without VSCC and VSCC (Figure 3B).

Discussion

The most important outcome of this study is the significant increase in methylation levels with severity of disease and clearly distinct methylation patterns in VIN with different cancer risk. VIN adjacent to VSCC revealed equally high methylation levels as VSCC. Contrarily, VIN without VSCC displayed a heterogeneous methylation pattern characterized by either low or high methylation levels, suggestive of a variable cancer risk. Our results demonstrate that DNA methylation of the 12 genes studied is associated with vulvar carcinogenesis, with highly comparable results for both HPV-induced and HPV-independent oncogenic pathways. Altogether, these methylation markers may provide valuable biomarkers for risk stratification of VIN.

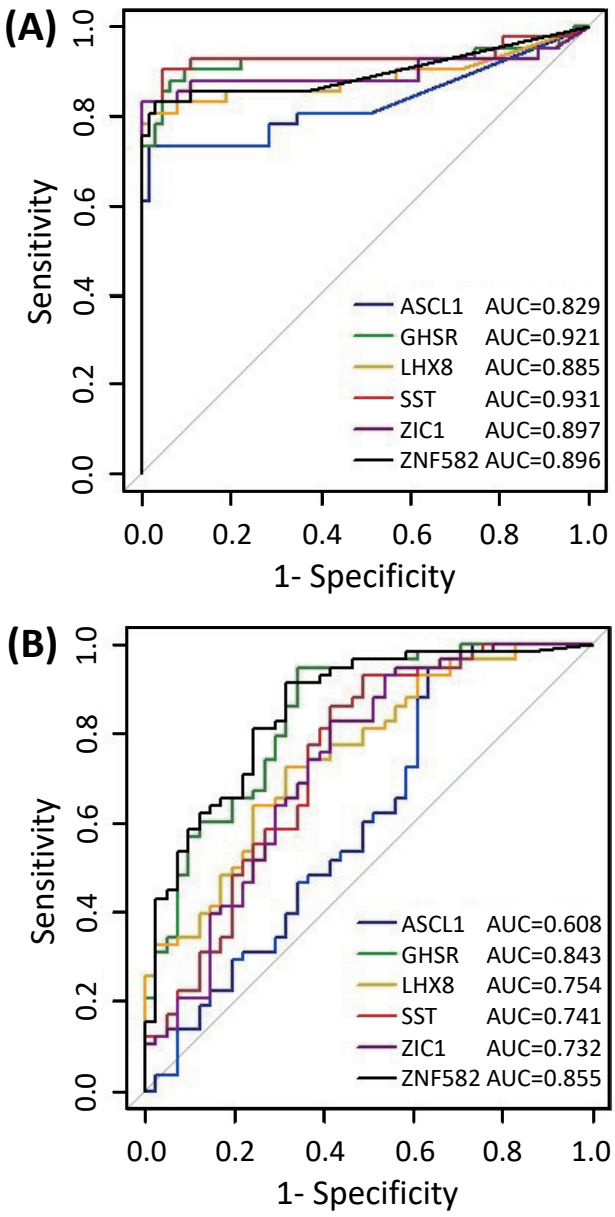

Figure 3. Diagnostic performance of six markers (*GHSR, SST, ZIC1, ASCL1, LHX8* and *ZNF582*) for the ability to distinguish VIN without VSCC from controls (A) and VIN without VSCC from VSCC (B), assessed by univariable logistic regression analysis and visualized with ROC curves and AUCs. AUC, area under the curve; ROC, receiver operating characteristics; VIN, high-grade vulvar intraepithelial neoplasia; VSCC, vulvar squamous cell carcinoma.

To our knowledge, this study examining 12 host-cell DNA methylation markers in 192 vulvar samples, including 41 well-defined VIN lesions without progression to VSCC during long-term follow-up and 30 VIN lesions adjacent to vulvar carcinoma, is the largest of its kind and the first to present results on these methylation markers in vulvar lesions. A correlation between increased methylation of specific markers and increasing severity of vulvar disease has already been described for a few other markers.[25-36] Only the markers *MGMT* and *p16*^{INK4a} have been investigated more than once. Methylation of *p16*^{INK4a} was commonly detected in both VIN and VSCC in six out of seven studies, while one study showed absence of *p16*^{INK4a} methylation in all 5 vulvar carcinomas studied.[26, 27, 29, 30, 32, 33, 35] *MGMT* methylation has been detected in 45% (13/20) and 36.7% (11/30) of vulvar carcinomas.[33, 37] In comparison, in our series 98.3% (57/58) of carcinomas showed a methylation-high pattern.

We have demonstrated that VIN adjacent to VSCC, considered as end stage VIN, displayed similarly high methylation levels as VSCC. It can be hypothesized that in VIN without VSCC high DNA methylation levels reflect a high cancer progression risk. The methylation-high patterns seen in a subset of VIN without VSCC, can be explained by the fact that VIN is usually not diagnosed until a late stage, when symptoms have already developed. Adequate treatment of such lesions may have prevented cancer development. The observed varying methylation patterns in VIN without VSCC is consistent with the molecular heterogeneity described for copy number alterations and gene expression profiles in VIN.[9] This molecular heterogeneity might in part explain why only a subset of VIN progress to cancer. Ideally, methylation biomarkers could guide clinical management with a more aggressive treatment for patients with VIN with many (epi)genetic alterations or methylation-high patterns, while more conservative strategies can be chosen for patients with VIN with low methylation levels. Clinical guidance by additional use of methylation biomarkers could therefore potentially decrease harms of treatment and associated psychosexual sequelae.[4]

Heterogeneous methylation patterns of the genes studied have also been described in other studies on anogenital disease.[10, 13, 38] In cervical scrapings of patients with cervical intraepithelial neoplasia grade 3 (CIN3) methylation levels were found to be linked to duration of disease existence, as was based on duration of the preceding high-risk HPV infection. More advanced CIN3 lesions, with a presumed high cancer progression risk, showed high methylation levels, equal to cervical cancers. On the other hand, so-called early CIN3 lesions with a lower risk of progression to cancer were generally characterized by low methylation levels.[11, 12, 38, 39] Similar findings have been described in high-grade anal intraepithelial neoplasia (AIN) of HIV-positive men having sex with men, also revealing heterogeneous methylation patterns with

a subset of high-grade AIN resembling anal cancer.[13, 19] In contrast to the methylation patterns seen in CIN or AIN, characterized by a gradual range of average predicted probabilities, predicted probabilities in VIN without VSCC were either low or high.[19, 38] The predicted probability model using VSCC samples as cases and healthy vulvar tissues as controls explains the dichotomy observed in our series.

One VSCC sample showed low individual predicted probabilities for 5 of 6 markers. This sample was HPV16 positive and was diagnosed in a 36-year old woman, which is a remarkably low age for vulvar cancer. Studies have described an age-associated increase in methylation levels.[40, 41] However, in our study we found increased methylation levels in both young and older patients and therefore solely age is unlikely to explain the low methylation pattern in this case.

All our markers showed a very good performance, indicated by high AUCs, for the distinction between VIN without VSCC and controls (AUC 0.829–0.931), and between VIN without VSCC and VSCC (AUC 0.608–0.855). These results may be biased by our sample selection and the composition of the disease categories, because disease category sizes were not corrected for actual disease prevalence. Accordingly, no conclusions regarding clinical performance or optimal marker combinations can be drawn yet.

Our study has multiple strengths. This is the largest study in terms of markers and sample size, covering the complete spectrum of vulvar neoplasia. Controls were collected from healthy women resulting in uniform low methylation levels. VIN adjacent to VSCC was used as surrogate for VIN with high cancer risk, which we believe is a first necessary step in the exploration of methylation biomarkers for risk stratification of VIN. Our results on VIN adjacent to VSCC demonstrate that high methylation levels are likely linked to VSCC development. Also, we demonstrated a good performance of our markers in both HPV-positive and -negative samples, in line with some of the markers also being methylated in other non-HPV-induced cancers.[42, 43]

Our study also has several limitations. Since we analyzed VIN adjacent to VSCC instead of VIN lesions showing progression to VSCC during follow-up, we cannot prove VIN with a methylation-high pattern do, indeed, have a higher risk of progression to cancer than their counterparts with a methylation-low pattern. Second, the majority of VIN without VSCC (i.e. 37/41) were HSIL, while only 4/41 were dVIN. The low number of dVIN in this group is explained by the fact that most dVINs are recognized at time of VSCC diagnosis and not prior to VSCC diagnosis. Third, due to DNA limitations not all markers could be tested on all samples. Nevertheless, a similar

trend in methylation levels per disease category was observed for all 12 markers. Fourth, across disease categories, median age of the patients differed, which might have influenced the methylation levels. However, the age in our series reflects age distribution seen in regular care.[5] Moreover, the effect of age on methylation levels is probably much weaker than the effect of strong biological processes involved in vulvar carcinogenesis.[41]

In conclusion, this study examining 12 DNA methylation markers revealed that methylation levels significantly increased from healthy vulvar tissue towards vulvar cancer. Histopathologically similar VIN without VSCC lesions displayed a heterogeneous methylation pattern. The methylation-high pattern found in a subset of VIN and VIN adjacent to VSCC indicates the promising value of host-cell DNA methylation testing to distinguish between VIN with low or high cancer progression risk. This is especially true for women with HSIL, in whom cancer risk stratification is clinically relevant. Next studies should include patients with VIN with variable clinical outcomes and long term follow-up data to further evaluate the potential value of these methylation biomarkers for cancer risk stratification.

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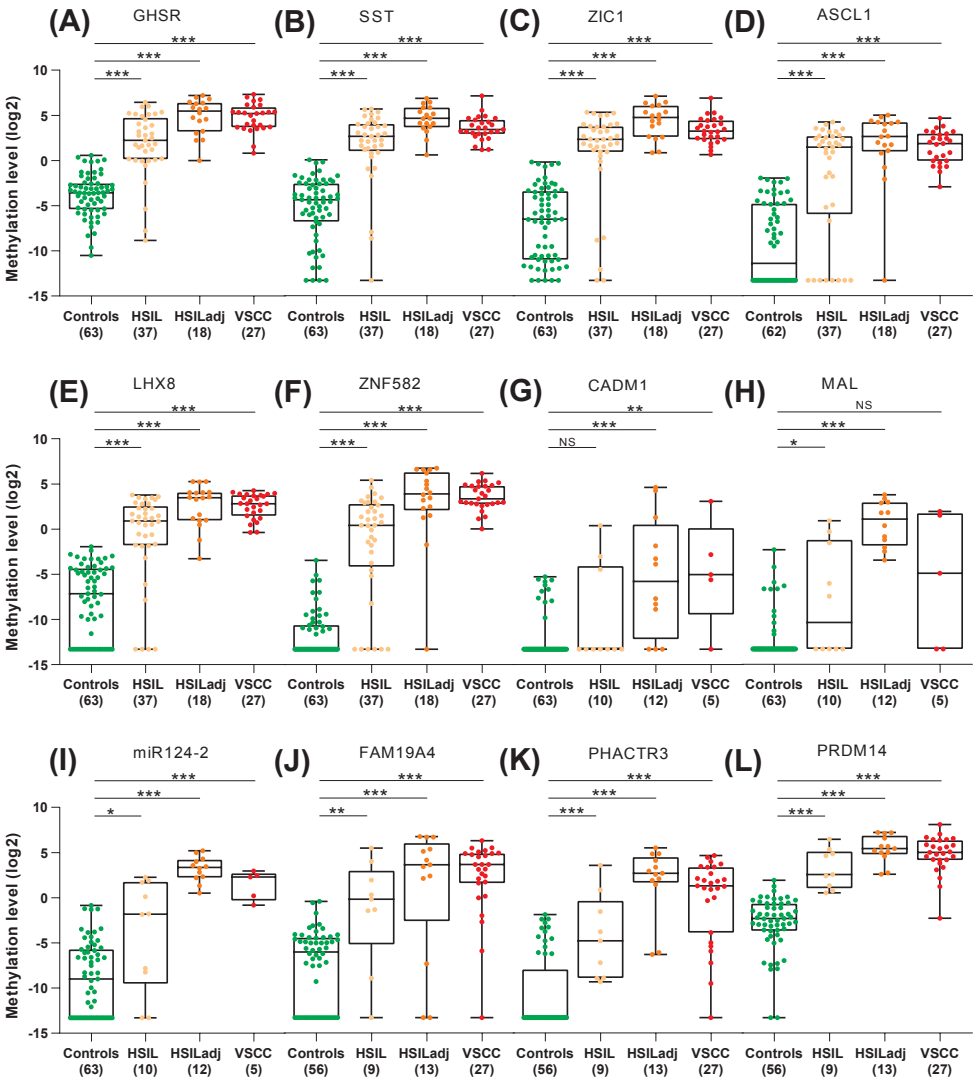
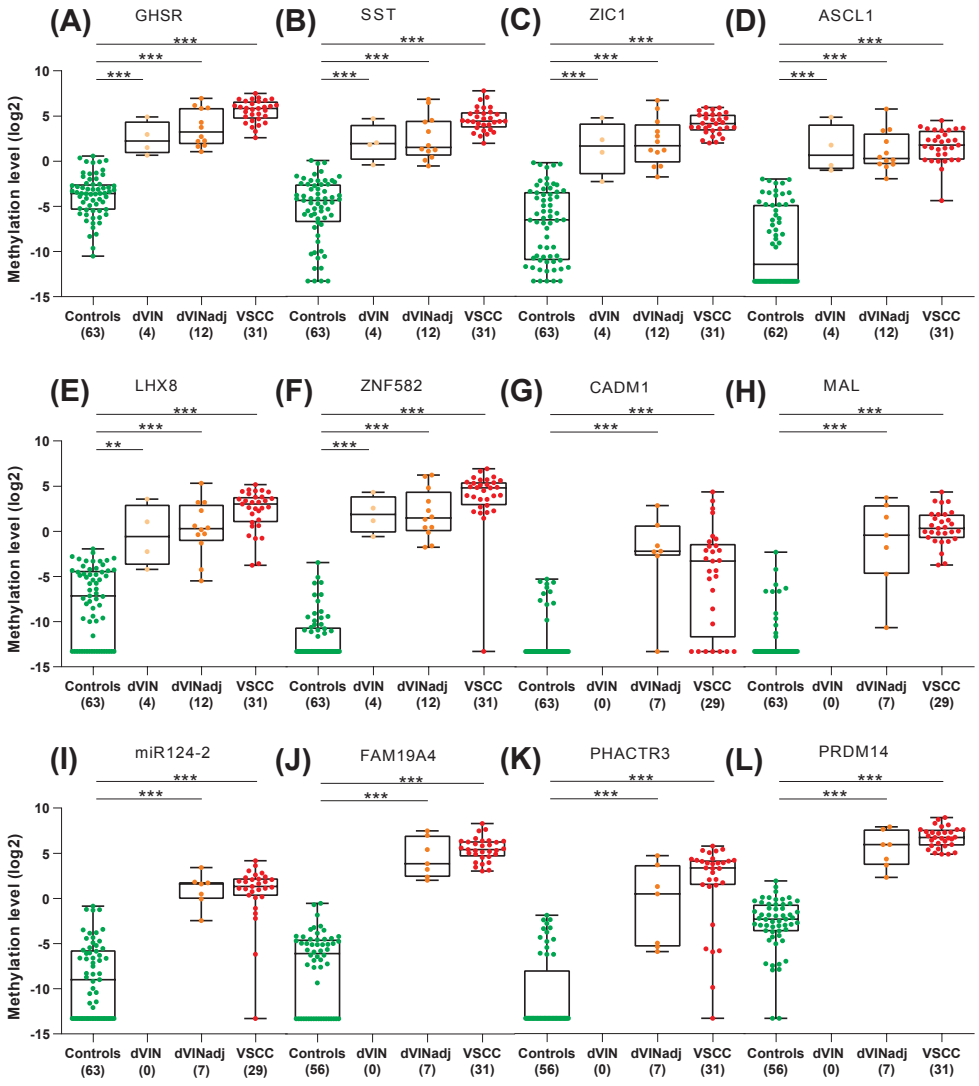


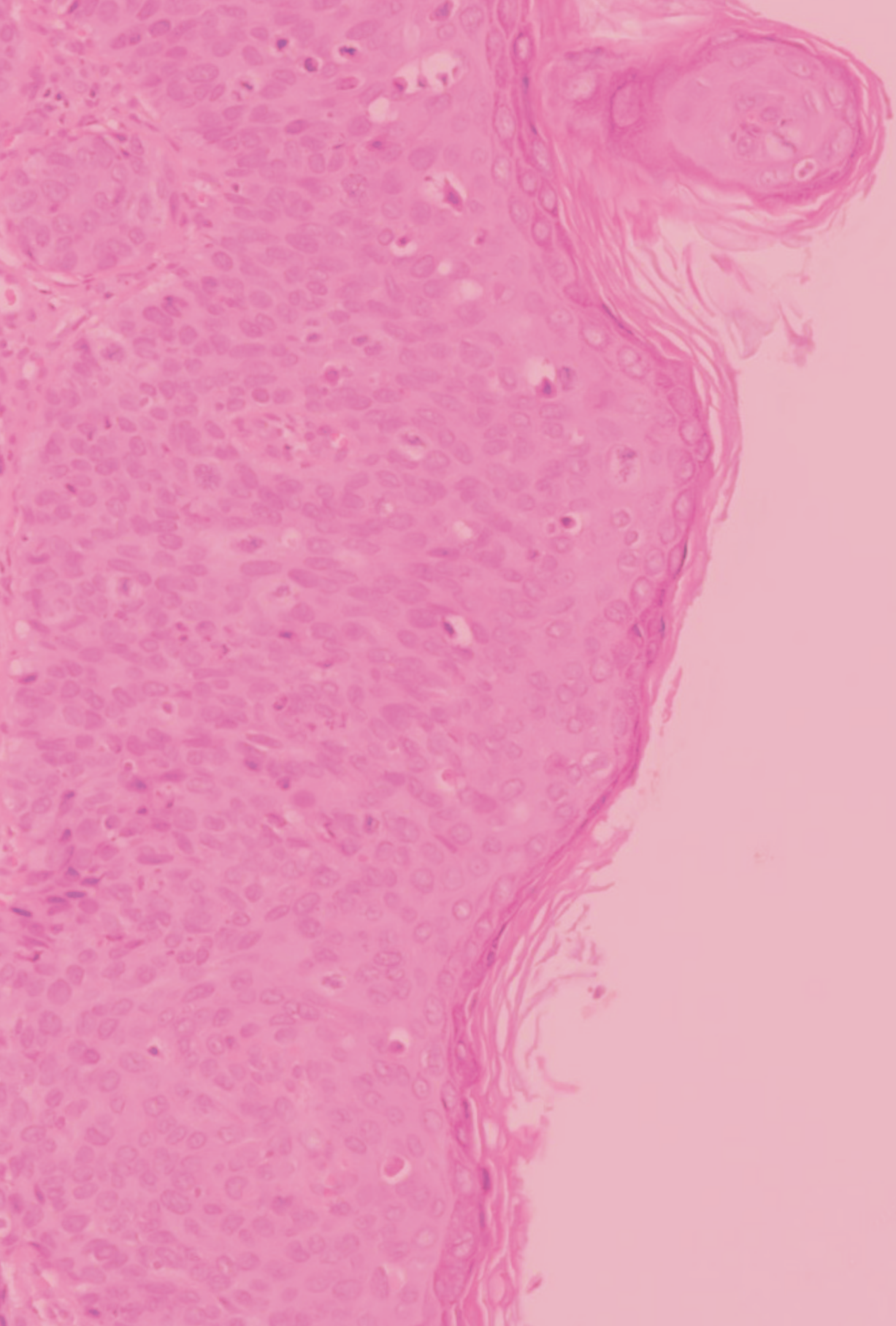
Supplementary Figure 1. DNA methylation levels shown relative to the reference gene ACTB (log2-transformed Δ(Δ)Ct ratios; y-axis) for HPV positive samples for the 4 disease categories (x-axis) for 12 markers: (A) GHSR, (B) SST, (C) ZIC1, (D) ASCL1, (E) LHX8, (F) ZNF582, (G) CADM1, (H) MAL, (I) miR124-2, (J) FAM19A4, (K) PHACTR3, and (L) PRDM14.

Differences between histological categories upon Kruskal-Wallis test, followed by post hoc testing using the Mann-Whitney U test and Bonferroni multiple testing correction: *p<.05 (marginal evidence), **p<.01 (moderate evidence), ***p<.001 (strong evidence), NS: not significant. Abbreviations: HSIL = high-risk squamous intraepithelial lesion, HSILadj = HSIL adjacent to VSCC, VSCC = vulvar squamous cell carcinoma



Supplementary Figure 2. DNA methylation levels shown relative to the reference gene ACTB (log2-transformed Δ(Δ)Ct ratios; y-axis) for HPV negative samples for the 4 disease categories (x-axis) for 12 markers: (A) GHSR, (B) SST, (C) ZIC1, (D) ASCL1, (E) LHX8, (F) ZNF582, (G) CADM1, (H) MAL, (I) miR124-2, (J) FAM19A4, (K) PHACTR3, and (L) PRDM14.

Differences between histological categories upon Kruskal-Wallis test, followed by post hoc testing using the Mann-Whitney U test and Bonferroni multiple testing correction: *p<.05 (marginal evidence), **p<.01 (moderate evidence), ***p<.001 (strong evidence), NS: not significant. Abbreviations: dVIN = differentiated high-grade vulvar intraepithelial neoplasia, dVINadj = dVIN adjacent to VSCC, VSCC = vulvar squamous cell carcinoma



CHAPTER 5

Biomarker expression in multifocal vulvar high grade squamous intraepithelial lesions

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Abstract

In patients with high-grade squamous intraepithelial lesion (HSIL) of the vulva, the presence of multiple lesions called multifocal HSIL is common. The aim of this study was to investigate biomarker expression profiles in multifocal HSIL. In total, 27 lesions from 12 patients with high-risk human papillomavirus (HPV)-positive multifocal HSIL were tested for HPV genotype, expression of $p16^{INK4a}$ and Ki-67, and DNA methylation of 6 genes. HPV16 was found most commonly in 21 (77.8%) HSILs. In 2 (16.4%) patients HPV genotype differed between the lesions. All lesions demonstrated diffuse $p16^{INK4a}$ staining of which 3 (11.1%) were combined with a patchy staining. One patient (8.3%) demonstrated markedly different DNA methylation levels between lesions. Generally, heterogeneity in methylation profiles was mainly observed between different patients, even when other biomarkers showed similar expression. In conclusion, this study is the first to demonstrate heterogeneity of individual lesions in patients with multifocal HSIL. The studied biomarkers have the potential to refine prognostic and predictive diagnostics. Future longitudinal studies are needed to further explore the potential of a biomarker profile for management of patients with multifocal HSIL.

Introduction

High-grade vulvar intraepithelial neoplasia (VIN) is the precursor of vulvar squamous cell carcinoma (VSCC). High-grade VIN is categorized into vulvar high-grade squamous intraepithelial lesion (HSIL), which is human papillomavirus (HPV)-associated, and differentiated VIN (dVIN), which is HPV-independent and associated with lichen sclerosis (LS)[1-3]. HSIL, also known as usual type of VIN (uVIN), is the most common type of VIN and occurs mainly in smoking patients aged 35 to 50 years[4]. The presence of multiple HSILs, a frequent finding at clinical examination, is called multifocal HSIL[5-7]. To confirm the clinical diagnosis and to exclude underlying invasive disease, multiple biopsies or a so-called vulvar mapping is frequently performed in patients with multifocal HSIL. Treatment options for vulvar HSIL vary from topical imiquimod to surgery, the latter often leading to somatic and psychosexual morbidity[8,9].

HPV infection is found in more than 80% of HSILs and HPV genotypes 16 and 18 are the most common identified[10,11]. Immunostaining of $p16^{INK4a}$ is often used as a surrogate marker of HPV-dependent high-grade intraepithelial lesions[12]. In recent years, DNA methylation of specific genes has shown a promising biomarker

in the identification of anogenital lesions, including vulvar neoplasia, being at risk for progression or cancer[13-15]. In vulvar neoplasia, it was shown that methylation levels increased with severity of disease, i.e. from control vulvar tissue through VIN to VSCC[16]. The expression of biomarkers in multifocal HSILs has never been studied before while this information may have predictive value with regard to the clinical course of individual lesions. Therefore, the aim of this study was to compare histopathological and molecular characteristics amongst individual lesions of patients with multifocal HSIL, i.e. HPV genotyping, immunohistochemical staining patterns of $p16^{INK4a}$ and Ki-67, and methylation profiles of 6 genes *GHSR, SST, ZIC1, ASCL1, LHX8* and *ZNF582*.

Materials and methods

Patients and samples

This study included 12 patients with multifocal vulvar HSIL. In total, 27 lesions, varying from 2 to 4 lesions per patient, were examined. Only baseline high-risk HPV positive HSILs were included, before treatment interference. Multifocal lesions were defined as multiple HSILs separated by unaffected vulvar skin. Confluent areas of HSIL were excluded. Tissues were selected from a historical cohort of patients with vulvar diseases including VIN, LS and VSCC, which has been described in detail previously[4,17]. Samples were collected from the Pathology archives of Amsterdam UMC, location VUmc and AMC, between 1991 and 2005. The selected tissues were anonymously processed for the purpose of this study. Clinical characteristics, i.e. smoking status, immunodeficiency, topographic site, lesional aspect, and the presence of other anogenital conditions, were extracted from a pseudonymized clinical database, using Castor EDC. Patient identity was protected by study-specific unique patient numbers. Accordingly, no further patient approval was needed. The local Medical Ethics Committee of Amsterdam UMC, location VUmc, confirmed that the Medical Research Involving Human Subjects Act did not apply to this study and approved the study under reference number 2017.561.

2.2 Processing of tissue blocks

For contamination-free DNA isolation, whole tissue sections of formalin-fixed, paraffin embedded (FFPE) tissue blocks were sectioned using the sandwich method. The first and last sections (3 µm) were used for hematoxylin–eosin (H&E) staining to ensure the presence of the lesion. In-between sections were collected in sterile PCR tubes for DNA isolation (10 µm) and for immunostaining (3 µm). Precautions were taken to avoid cross-contamination as described before[18].

Histopathology and immunohistochemistry of p16^{INK4a} and Ki-67

All H&E and immunohistochemically stained slides were scored by a gynecopathologist (M.C.G.B.) and a senior resident in pathology (N.B.T.). The Optiview detection kit with the automated 100 BenchMark ULTRA IHC/ISH system (Roche) was used to perform immunostaining of both p16^{INK4a} and Ki-67. Mouse monoclonal antibodies against the p16^{INK4a} antigen (clone E6H4; Roche, Basel, Switzerland) were used for immunostaining of p16^{INK4a}. Immunostaining of Ki-67 was performed with mouse monoclonal antibodies against the Ki-67 antigen (clone Ki-67; Dako, Glostrup, Denmark). A negative or patchy staining pattern of p16^{INK4a} was scored as 0, a diffuse (or block) p16^{INK4a} staining pattern up to the lower third of the epithelium as score 1, extending above the lower third of the epithelium as score 2, or extending more than two-thirds of epithelium as score 3. When a diffuse staining pattern for p16^{INK4a} was present, it was scored whether this pattern was completely diffuse, or combined with a negative or patchy staining pattern. Ki-67 expression was scored as not increased (score 0), increased in the lower third (score 1), increased in the lower two-thirds (score 2) or increased in more than two-thirds (score 3) of the epithelium.

DNA isolation

The in-between sections were used for DNA isolation using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted with the easyMAG 3 elution buffer (bioMérieux, Boxtel, the Netherlands). DNA concentrations were measured using Qubit (Thermo Fisher Scientific Inc, Qiagen).

DNA methylation analysis

For methylation analysis, isolated DNA was bisulphite-converted using the EZ-DNA Methylation kit (Zymo Research, Orange, CA, USA)[19]. Methylation analysis was performed using EpiTect MethyLight Master Mix (Qiagen, Hilden, Germany), together with fluorescent dry-labelled probes, 50 ng of bisulphite-converted DNA and 100-300 nM of each primer[20]. Six methylation markers, *GHSR, SST, ZIC1, ASCL1, LHX8* and *ZNF582*, and the reference gene, β-actin (ACTB), were tested by quantitative methylation-specific PCR (qMSP) assays as described previously[20,21]. Samples with a quantification cycle threshold (Ct) of ACTB ≤32 indicated sufficient DNA and adequate bisulphite conversion[21]. No invalid test results were obtained. ΔCt ratios were computed using the comparative Ct method, normalizing target Ct values to ACTB[22]. Additionally, DNA methylation levels for all genes were categorized into 4 quartiles: ≤25th percentile, >25th ≤ 50 percentile, >50th ≤ 75 percentile, and >75th percentile.

2.6 Human papillomavirus (HPV) testing and genotyping

The QIAscreen® HPV PCR Test (QIAgen, Hilden, Germany) was used to perform high-risk HPV DNA-testing, according to the manufacturer's instructions. Analysis was directed against the E7 gene of the following high-risk HPV genotypes, i.e. 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68, with partial genotype information (HPV16 and -18)[23]. β-globin was used as internal quality control.

Results

Baseline characteristics

Baseline characteristics of the study population are shown in Table 1. Median age was 40 years (range 24-58). In total, 27 lesions of 12 patients with multifocal HSIL were analyzed, varying from 2 to 4 lesions per patient. Aspects of lesions, including shape, color and thickness, had been documented in the records of 7 patients. Topographic sites included labia minora (n=7), labia majora (n=7), perineum (n=3), commissura posterior (n=2), perianal region (n=1), and clitoris (n=1). Of all 12 patients, 8 (66.7%) patients had other HPV related anogenital conditions, i.e. multicentric disease (squamous intraepithelial lesions of cervix, vagina, or anus), and/or anogenital condylomata acuminata. Two patients (16.7%) were immunocompromised, one by human immunodeficiency virus (HIV) and one by systemic lupus erythematosus (SLE). None of the patients had vulvar LS.

Table 1. Baseline characteristics of the study population of 12 patients with multifocal HSIL

						Baseline
Patient	**Age (years)**	**Other anogenital conditions**	**Smoking**	**Immuno-compromised**	**Number**	**Aspect**
1	42	AIN3, condylomata acuminata	Unknown	Unknown	2	Not specified
2	24	None	Yes	No	4	Hyperpigmentation, maculopapulous
3	44	AIN2	Yes	No	2	Leukoplakia
4	37	CIN3	Yes	No	2	Not specified
5	58	AIN3, CIN3	Unknown	No	2	Hyperpigmentation, condylomatous brown
6	38	None	Yes	No	2	Not specified
7	45	CIN3, condylomata acuminata	Yes	No	2	Hyperpigmentation
8	31	CIN2, VAIN2, condylomata accuminata	Yes	Yes	2	Not specified
9	44	None	Unknown	Unknown	2	Hypertrophic dystrophic
10	49	CIN3	Yes	No	3	Papillomatous, erosive, varyingly pigmented
11	38	Unknown	Unknown	Yes	2	Not specified
12	28	CIN2, condylomata acuminata	Yes	No	2	Hyperpigmentation, condylomatous brown

* cured after primary treatment

Abbreviations: AIN = anal intraepithelial neoplasia, CIN = cervical intraepithelial neoplasia, VAIN = vaginal epithelial neoplasia, lab maj = labium majus, lab min = labium minus, R = right side, L = left side.

Baseline			Follow-up			
Topography	**Type of biopsy**	**Primary treatment**	**Time to last HSIL diagnosis (years)**	**VSCC during follow-up**	**Time to VSCC (years)**	**Topography VSCC**
Perineum, lab maj R	Diagnostic	Skinning vulvectomy	*	No	NA	NA
Lab maj R and L, lab min R and L	Diagnostic	Skinning vulvectomy	0.8	No	NA	NA
Lab min R and L	Diagnostic	Local excision	18.5	No	NA	NA
6 and 9 o'clock	Diagnostic	Laser evaporatisation	16.7	No	NA	NA
Lab min R and L	Diagnostic	None	0.5	No	NA	NA
Perineum, lab maj/min R	Therapeutic	Local excision	20.2	No	NA	NA
Commisura posterior, perianal	Therapeutic	Local excision	14.8	Yes	9.2	Perianal
Lab maj/min L, lab min R	Diagnostic	Local excision	*	No	NA	NA
Lab maj R and L	Diagnostic	Local excision	3.1	No	NA	NA
Perineum, lab maj/min L, lab min R	Diagnostic	Skinning vulvectomy	19.1	Yes	9.3	Perianal
Lab min R, commissura posterior	Therapeutic	Local excision	11.4	Yes	5.9	Anterior L
Lab maj/min L, clitoris	Therapeutic	Skinning vulvectomy	16.1	Yes	11.4	Posterior L

Biomarker expression

The histopathological and biomarker characteristics of all 27 HSILs are shown in Figure 1. Genotyping showed that HPV16 was most common, found in 21 HSILs (77.8%). One of the 27 lesions was HPV18 positive (3.7%). Two patients had a different HPV genotype in each HSIL (16.7%, patient 5 and 7). One patient had multiple HPV genotypes in one HSIL (patient 8, lesion 1). All HSILs showed diffuse $p16^{INK4a}$ staining in two thirds or more of the epithelium (score 2 or 3, respectively). In the majority of HSILs (n=24, 88.9%) a completely diffuse staining pattern for $p16^{INK4a}$ was found while 3 HSILs (11.1%) showed a combined diffuse and patchy pattern (Figure 2). All HSILs showed increased proliferation activity up to two thirds or more of the epithelium (score 2 or 3, respectively), measured by Ki-67 expression. DNA methylation levels between HSILs varied from absent (i.e. log2 transformed methylation level of -13.29) to high (methylation level of 6.75). Only one patient (patient 7) showed marked methylation differences between the HSILs, with a difference in DNA methylation of at least two quartiles in most (5 out of 6) markers. The remaining 11 patients had smaller differences in methylation levels or differences in only a few markers between HSILs. Interestingly, all 3 HSILs with combined diffuse and patchy $p16^{INK4a}$ staining showed lower methylation levels compared to their counterpart HSIL with only diffuse $p16^{INK4a}$ staining (patient 7, 8 and 12). Both in HPV16 and non-HPV16 low and high methylation levels were seen and no statistical significant difference in methylation levels between HPV16 and non-HPV16 HSIL was observed. Overall, DNA methylation levels showed a trend towards increased methylation levels with higher $p16^{INK4a}$ expression, but given the low numbers, results were not significant.

	Patient 1		Patient 2				Patient 3		Patient 4	
Lesion	1	2	1	2	3	4	1	2	1	2
HPV	16	16	16	16	16	16	other	other	16	16
p16^{INK4a}	2	2	3	2	2	2	2	2	3	2
Ki-67	2	2	3	2	2	3	2	3	3	3
GHSR	-0.34	2.10	-1.41	-2.72	-2.40	-4.08	1.26	2.26	-1.01	1.94
SST	2.06	5.58	-0.23	-2.85	-3.14	-4.59	4.22	5.89	1.76	2.46
ZIC1	2.23	4.63	-13.29	-5.42	-13.29	-13.29	1.79	-0.04	1.34	0.54
ASCL1	0.35	-0.73	-13.29	-13.29	-4.84	-13.29	-1.55	0.97	3.25	0.62
LHX8	1.60	2.83	-13.29	-13.29	-0.91	-3.81	-1.00	4.69	-0.98	1.34
ZNF582	-13.29	5.91	-2.85	-13.29	-13.29	-13.29	-2.44	0.42	3.37	-1.44

	Patient 5		Patient 6		Patient 7		Patient 8	
Lesion	1	2	1	2	1	2	1	2
HPV	16	18	other	other	other	16	16, other	16
p16^{INK4a}	3	2	2	3	3	3	2	2
Ki-67	2	2	2	3	3	3	3	3
GHSR	1.99	-0.86	1.27	2.91	-0.03	3.02	-0.72	-2.45
SST	0.17	-1.14	-1.60	3.73	0.39	2.28	-0.12	-7.93
ZIC1	0.52	0.59	0.40	-0.89	-10.41	3.55	-0.87	-13.29
ASCL1	-13.29	-13.29	2.44	1.09	-2.60	2.78	-2.50	-13.29
LHX8	-13.29	-13.29	3.16	3.07	-2.85	3.40	0.40	-13.29
ZNF582	-4.54	-6.60	-13.29	-0.48	-13.29	3.54	-0.82	-13.29

	Patient 9		Patient 10			Patient 11		Patient 12	
Lesion	1	2	1	2	3	1	2	1	2
HPV	16	16	16	16	16	16	16	16	16
p16^{INK4a}	2	2	3	2	2	3	3	3	3
Ki-67	2	2	3	2	2	3	3	3	3
GHSR	2.97	4.45	0.16	2.83	-0.14	0.27	6.48	-4.55	-2.11
SST	4.84	4.66	2.67	1.81	-0.06	3.85	6.66	-3.27	-1.98
ZIC1	4.35	4.16	-0.23	-1.00	-13.29	4.62	6.75	-6.76	-4.82
ASCL1	1.69	1.37	0.03	-3.35	-2.25	1.21	3.62	-13.29	-13.29
LHX8	3.75	3.85	2.58	-0.36	0.60	-0.25	5.60	-13.29	-13.29
ZNF582	4.40	4.52	3.85	-2.42	-13.29	4.47	5.67	-13.29	-13.29

Legend					
p16^{INK4a}	negative/patchy (0)	lower 1/3 (1)	lower 2/3 (2)	full thickness (3)	combined
Ki-67	normal (0)	lower 1/3 (1)	lower 2/3 (2)	full thickness (3)	
Methylation	≤25th perc.	>25th ≤50th perc.	>50th ≤75th perc.	>75th perc.	

Figure 1. Histopathology and biomarker profiles in 12 patients with multifocal HSIL

Immunohistochemical scores for p16^{INK4a} (0-3) and Ki-67 (0-3), high-risk HPV genotypes and log2 transformed methylation levels of all HSILs. DNA methylation levels for all genes (*GHSR, SST, ZIC1, ASCL1, LHX8, ZNF582*) were categorized into 4 quartiles. Each column represents one HSIL. The colours refer to the biomarker expression, as indicated in the legend. Abbreviations: HPV = human papillomavirus.

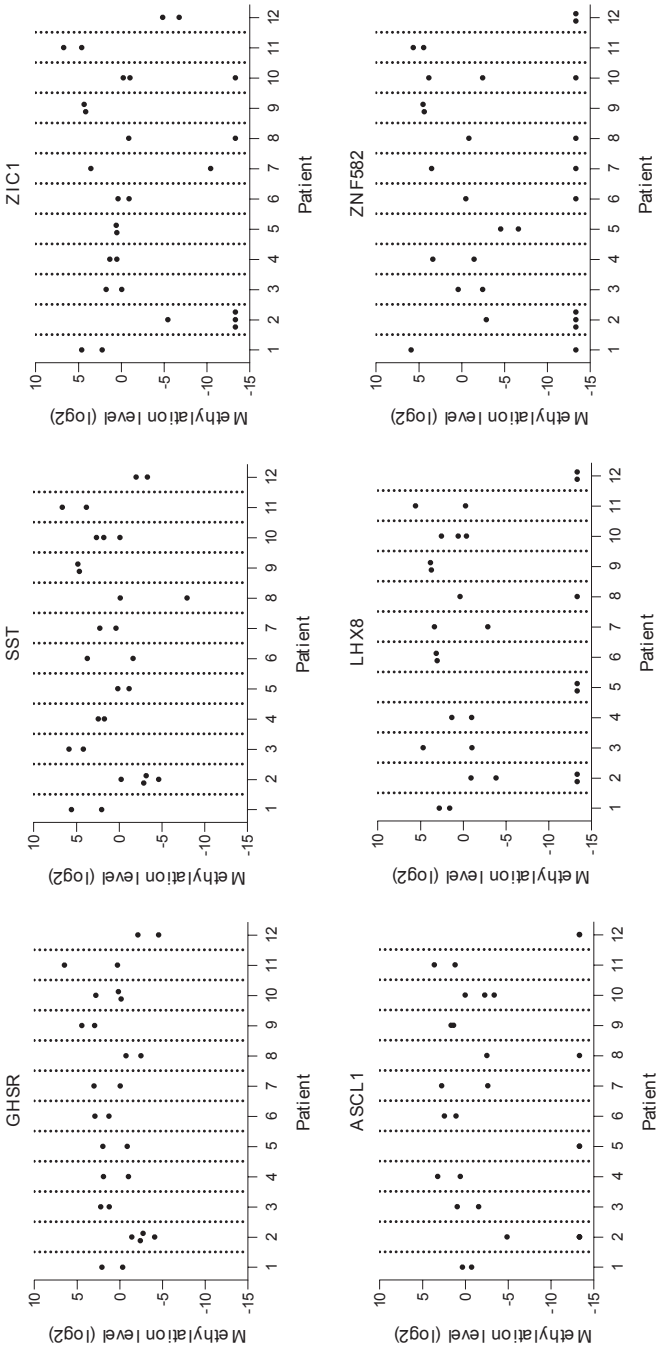

Figure 2. Methylation levels per lesion per patient for all six genes (*GHSR, SST, ZIC1, ASCL1, LHX8, and ZNF582*)

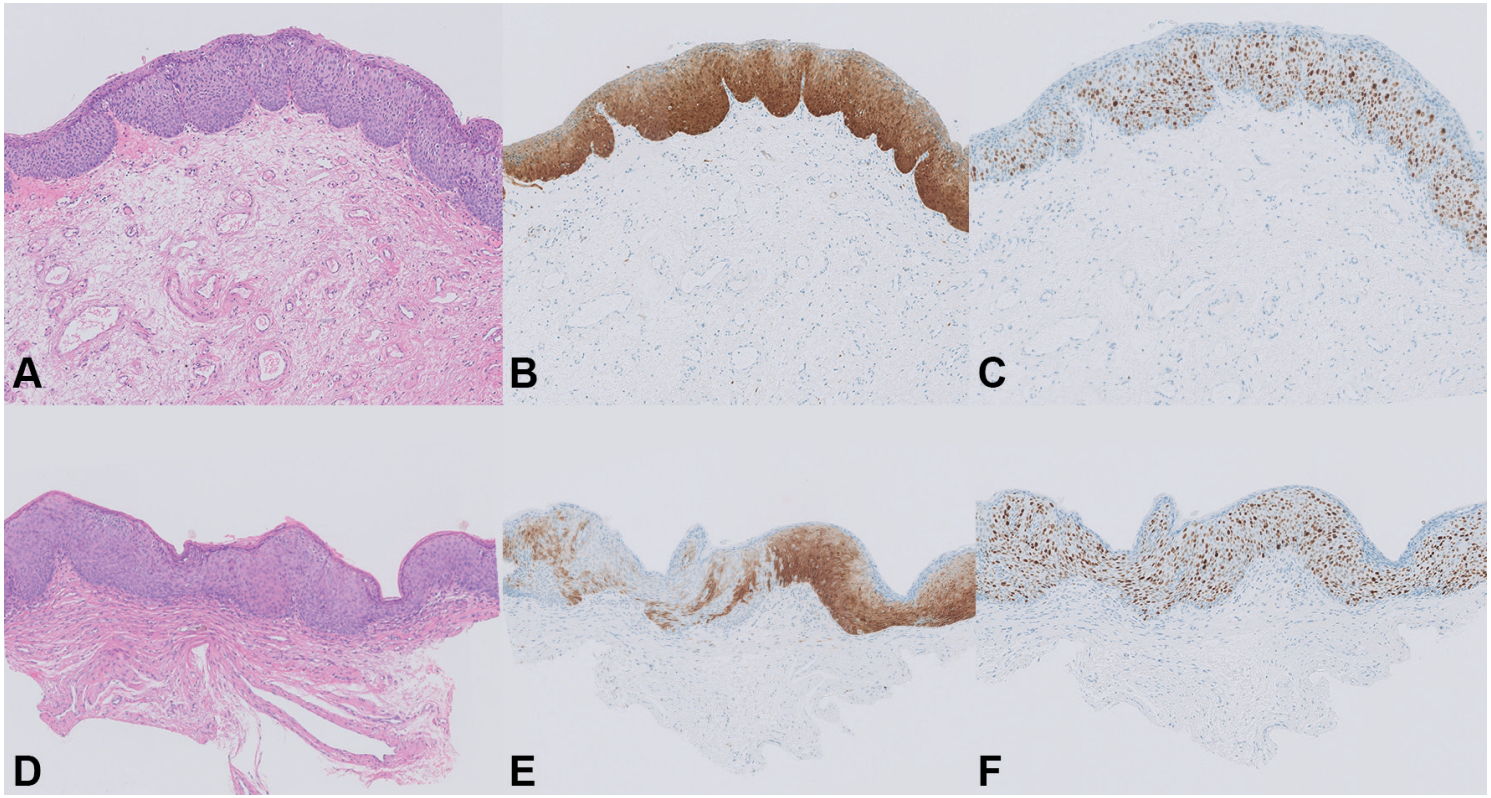

Figure 3. Staining patterns for p16^{INK4a} in HSIL

Patient 8, HSIL 1: **(A)** H&E stain; **(B)** Homogeneous p16^{INK4a} staining, score 3; **(C)** Full thickness immunostaining of Ki-67, score 3.

Patient 8, HSIL 2: **(D)** H&E stain; **(E)** Combined diffuse and patchy p16^{INK4a} staining, score 2; **(F)** Full thickness immunostaining of Ki-67, score 3.

Discussion

This study is the first to have systematically investigated the biomarker expression in individual vulvar high-grade lesions of patients with multifocal HSIL. For most patients with multifocal HSIL, the biomarkers showed comparable expression profiles between lesions. In one patient remarkable differences in DNA methylation levels of 5 out of 6 markers and in HPV genotype were observed, while both HSIL morphology and $p16^{INK4a}$/Ki-67 staining patterns were similar.

In patients with HSIL, it is often not possible to reliably diagnose HSIL based on only the clinical aspect of the lesion. The clinical features of HSIL vary in vulvar topography, size, surface, shape, color, and thickness[24]. Therefore, all clinically suspicious lesions are biopsied to confirm the diagnosis and to exclude invasive disease. This results in relatively high diagnostic costs and increased morbidity of patients. Testing for biomarkers might provide objective prognostic and predictive information, which is valuable for the management of patients with HSIL.

Of the 27 high-risk HPV positive HSILs, 78% had HPV16, 3.7% had HPV18, and 22% had another high-risk type. This distribution of HPV genotype is comparable to the literature[10,11]. Two of twelve patients had a different HPV genotype in each HSIL, indicating that these lesions developed independently. One patient had two high-risk HPV genotypes within the same lesion. According to the literature, more than 90% of VIN lesions is attributable to only one HPV genotype[10]. The presence of two HPV types in one lesion may result from a collision of two independent HSILs, each with an unique HPV type. However, the two HPV types detected in one lesion in the present study differed largely in abundance, with highly abundant HPV16 most likely being the single causative type. The low abundance of HPV-other may be explained by the patient being immunocompromised by systemic lupus erythematodes[25]. In cervical lesions, the presence of multiple HPV genotypes is thought to be associated with high-risk, persistent HPV infections, which is probably related to impaired immunity[26-28]. The biological relevance of different and multiple HPV genotypes in vulvar lesions has not been studied and remains to be elucidated. Also no data exist on progression risk in HSIL stratified per HPV genotype. In cervical premalignant lesions the progression risk is highest for HPV16[29]. In our study, no statistical significant difference in methylation levels between HPV16 and other high-risk HPV types was observed, likely due to small study numbers.

Consistent with a diagnosis of high-risk HPV-associated HSIL, all lesions stained diffusely positive for $p16^{INK4a}$ and showed increased Ki-67 expression. $P16^{NK4a}$

is frequently used to optimize grading of HPV-induced anogenital lesions and diffuse staining is considered a surrogate marker for HPV-associated high-grade anogenital lesions. The Lower Anogenital Squamous Terminology (LAST) Project only recommends the use of $p16^{INK4a}$ to differentiate between HSIL and LSIL or mimics of precancer[30]. In our study, 3 of 27 HSILs had a combined diffuse and patchy staining for $p16^{INK4a}$. While it is not clear whether this reflects the biological behavior of these lesions, it can be speculated that these HSILs have a lower malignant potential. Indeed, the lower malignant potential is supported by the very low or negative methylation levels found in these lesions. However, the far majority of HSILs demonstrated a uniform diffuse $p16^{INK4a}$ staining pattern while both high and low methylation levels were seen, indicating heterogeneity of vulvar HSILs. This observation is in agreement with our earlier studies showing that morphological identical vulvar HSILs show substantial molecular heterogeneity with respect to both copy number aberrations (CNA) and DNA methylation, despite similar histopathological classification and $p16^{INK4a}$/Ki-67 staining patterns[31,32]. This heterogeneity is also seen in cervical and anal $p16^{INK4a}$ positive HSIL, with a subset of those high-grade anogenital lesions having as high methylation levels as cancer[20].

This study has some limitations. The retrospective study design hindered collection of all patient characteristics, disabling us to link clinical characteristics to the biomarker expression of lesions. Secondly, the study population was too small to draw firm conclusions or to evaluate biomarker results in a multivariate analysis. Thirdly, since we analyzed a cross-sectional series of multifocal HSILs, we could not prove that multifocal HSILs with high expression levels had a higher risk of persistence or progression to cancer compared to multifocal HSILs with low expression levels. Further research on the role of the selected biomarkers in multifocal HSILs during the longitudinal course of vulvar carcinogenesis is needed.

Our study also has several strengths. It is the first to have systematically described the expression of multiple biomarkers, including HPV genotyping, immunohistochemistry and DNA methylation in patients with multifocal vulvar HSIL. Also, we were the first to show heterogeneity of vulvar HSILs. The studied biomarkers have great potential to refine prognostic and predictive diagnostics.

Conclusions

In conclusion, although only present in a small proportion of patients with multifocal HSIL, this study demonstrates that heterogeneity between individual lesions of patients with multifocal HSIL does exist. Future longitudinal studies are warranted to verify the potential of a biomarker profile for management of patients with multifocal HSIL at risk for developing vulvar cancer.

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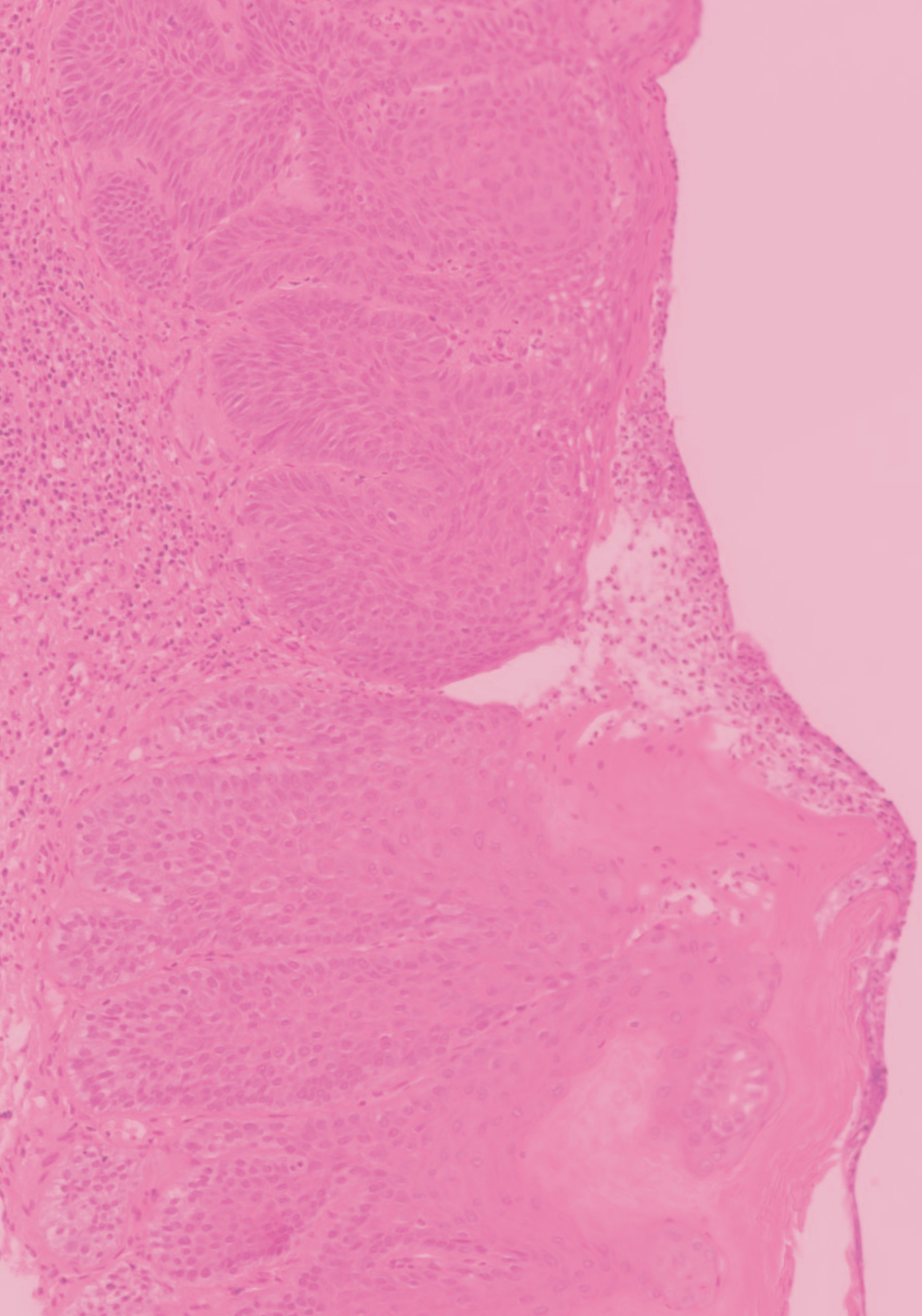

CHAPTER 6

High-grade vulvar intraepithelial neoplasia: comprehensive characterization and long-term vulvar carcinoma risk

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Abstract

Adequate diagnosis of human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesion (HSIL) and HPV-independent vulvar intraepithelial neoplasia (VIN) is essential but can be challenging. We comprehensively characterized a large population-based series of vulvar lesions, originally reported as high-grade VIN, and assessed the cancer risk.

Baseline high-grade VIN of 751 patients were categorized by histopathological reassessment, integrating the results of immunohistochemistry ($p16^{INK4a}$, p53, ki-67) and HPV DNA testing. Integrated analyses resulted in 88.4% HPV-associated lesions (77.0% HSIL, 10.9% LSIL and 0.4% VSCC), 10.9% HPV-independent lesions (6.1% HPV-independent VIN, 4.7% non-dysplastic lesions, and 0.1% VSCC) and 1.1% inconclusive lesions. HSIL demonstrated $p16^{INK4a}$ block-positivity in 99.0%, increased ki-67 in $\geq 2/3^{rd}$ of the epithelium in 93.6%, and HPV positivity in 99.6%. In HSIL, a p53 wild-type mid-epithelial staining pattern was common (51.6%) while this was not observed in HPV-independent lesions. HPV-independent VIN harboured mutant p53 patterns in 65.2% and showed a wide morphological spectrum, ranging from differentiated to non-differentiated ('HPV-associated-like', in 41.3%). Kaplan-meier analyses showed a 10-year cancer risk of 8.0% in HPV-associated HSIL, 67.4% in HPV-independent VIN/p53mutant, and 27.8% in HPV-independent VIN/p53wild-type. Strikingly, the 10-year cancer risk was 73.3% in HPV-independent VIN with non-differentiated ('HPV-associated-like') morphology.

Immunohistochemistry by $p16^{INK4a}$ and p53 is highly recommended for optimal categorization into HPV-associated and HPV-independent VIN, which is of utmost importance given the different cancer risk. The high cancer risk of HPV-independent VIN underscores the need for surgical treatment and close follow-up, especially in case of a p53 mutant pattern and/or non-differentiated morphology.

Introduction

Vulvar intraepithelial neoplasia (VIN), the precursor of vulvar squamous cell carcinoma (VSCC), is categorized into HPV-associated high-grade squamous intraepithelial lesion (HSIL) and low-grade SIL (LSIL), and HPV-independent VIN.(1) HPV-associated SIL occurs mainly in younger women and is treated by imiquimod, excision, or laserevaporiazation.(2, 3) HPV-independent VIN, often referred to as differentiated VIN (dVIN), occurs mainly in older women in a background of lichen sclerosus (LS) or lichen planus and is treated by excision.(2, 4) HPV-independent VIN often has a history of vulvar cancer or is diagnosed adjacent to cancer.(5, 6)

In contrast to HPV-associated SIL, HPV-independent VIN shows a wide spectrum of clinical and histomorphologic features, some overlapping with reactive/non-dysplastic dermatoses.(7-9) Given the high cancer risk in HPV-independent VIN, misclassified lesions can have serious clinical consequences. For optimal typing and grading of VIN, a few immunohistochemical (IHC) markers have been established. The Lower Anogenital Squamous Terminology (LAST) recommends the use of $p16^{INK4a}$ to differentiate between HSIL and LSIL.(10) To diagnose HPV-independent VIN, the use of p53 IHC can be helpful. However, caution is needed as approximately one third of HPV-independent VIN lacks a mutant p53 pattern while 'mutant-like' patterns, such as wild-type staining with markedly reduced staining intensity mimicking mutant 'null' staining, and wild-type mid-epithelial staining with basal sparing, mimicking mutant positive staining, can be seen in HPV-associated lesions.(11, 12)

The aim of this study was to categorize 751 vulvar lesions originally diagnosed as high-grade VIN (hg-VIN) into HPV-associated or HPV-independent categories by integrated analyses of histopathologic review, IHC results and HPV DNA testing, and to determine cancer risk for different subgroups of hg-VIN.

Materials and Methods

Study population

From a population-based historical cohort, a total of 894 patients diagnosed with hg-VIN (originally 884 HSIL and 12 HPV-independent VIN) between 1991 and 2011 were identified, as described previously.(6) Patients with prior or concurrent (i.e., within three months) VSCC were not included. Formalin-fixed, paraffin embedded tissue blocks of the baseline hg-VIN were retrieved. In order to determine progression to cancer, follow-up data were collected up to 2020, as previously described.(6)

This study was approved by the local Medical Ethics Committee of Amsterdam UMC, location VUmc. Informed consent was not required.

Categorization of vulvar lesions

Categorization was based on histopathological assessment by two pathologists (M.C.G.B., N.B.T.) with integrated analyses of IHC and HPV results. Vulvar lesions were categorized as HPV-associated (VSCC, HSIL, LSIL) or HPV-independent (VSCC, HPV-independent VIN, non-dysplastic lesions, including LS, reactive lesions and other non-dysplastic dermatoses). For HPV-independent VIN, differentiated and non-differentiated ('HPV-associated-like') morphology was recorded. Non-differentiated ('HPV-associated-like') morphology included lesions mimicking HSIL or LSIL, as described by Rakislova et al.(13) In short, non-differentiated ('HPV-associated-like') morphology represented all morphologies without epithelial differentiation characterizing 'classical' dVIN. This included both basaloid morphology, consisting of full-thickness epithelial atypia and high nuclear-to-cytoplasmic ratio, and the remainder of non-differentiated morphologies, mainly comprising papillary epithelium (whether or not inverted) with elongated, bulbous rete ridges, moderate to marked pleomorphism and koilocytic-like changes.(13, 14) Adjacent to areas of 'HPV-associated-like' HPV-independent VIN', more typical areas of dVIN could be seen.

Tissue processing

Details of tissue processing, IHC of $p16^{INK4a}$, p53 and ki-67, DNA isolation and HPV DNA testing are described in Supplementary File 1.

Immunohistochemical staining patterns of $p16^{INK4a}$, p53 and ki-67

Examples of the IHC staining patterns are presented in Figure 1. $P16^{INK4a}$ staining was scored as negative (absent or patchy) or block (diffuse) positive (≤1/3, ≤2/3, >2/3).(15) P53 staining was scored as wild-type (scattered or mid-epithelial with basal sparing) or mutant (nuclear positive including basal aberrant and parabasal/diffuse aberrant, null or cytoplasmic positive). A mutant positive staining pattern included the earlier described categories of 'basal overexpression' (i.e. uniformly strong nuclear staining in at least 80% of the basal cells without significant parabasal staining) and 'parabasal/diffuse overexpression' (i.e. uniformly strong nuclear staining of both the basal and the parabasal cells).(16, 17) Ki-67 staining was scored as not increased (a few positive parabasal nuclei) or increased (≤1/3, ≤2/3, >2/3).(15) In addition, there was a so-called 'viral' ki-67 staining pattern when there was increased staining in the upper layers with less or no increased staining in the lower layers.

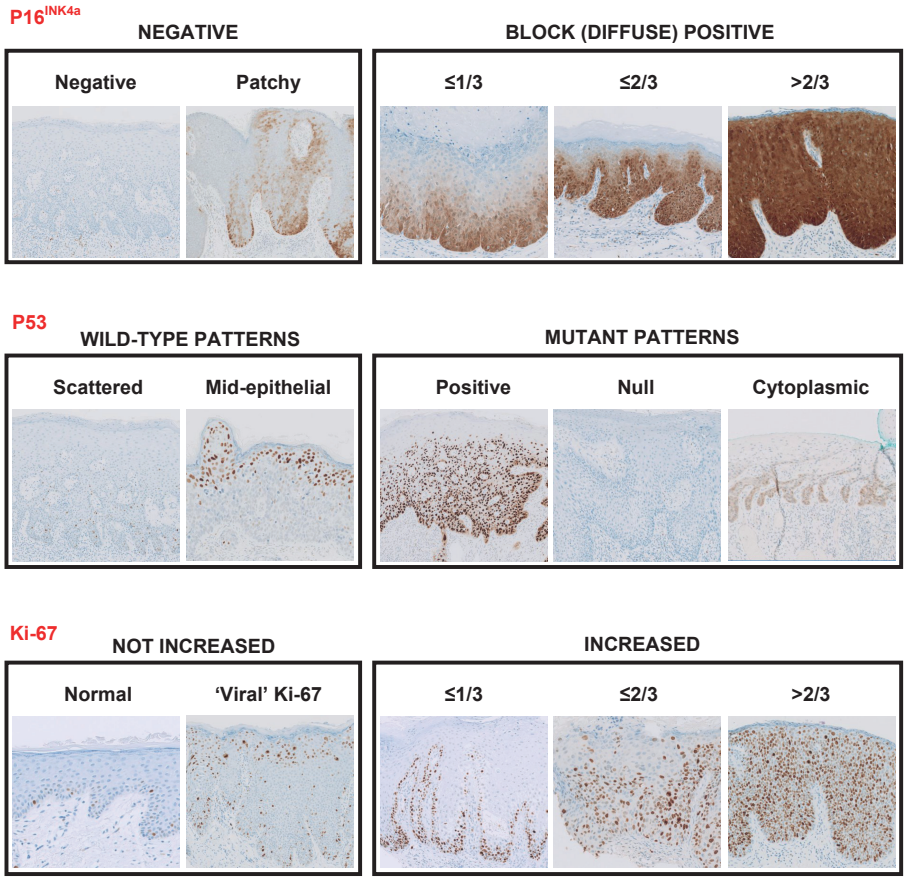


Figure 1. Representative examples of p16^{INK4a}, p53 and ki-67 immunohistochemical staining patterns. The p16, p53 and the increased ki-67 staining patterns haven been described before.(15-17) The p53 mutant positive pattern includes the earlier described patterns of 'basal overexpression' and 'parabasal/diffuse overexpression'.(16, 17)

Human papillomavirus (HPV) genotyping

HPV DNA testing was performed for high-risk (hr) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 ('possibly carcinogenic'), and 68 ('probable carcinogenic').(18) In case a lesion was assumed to be HPV-associated after histopathological assessment and tested negative for hr-HPV DNA, additional testing for low-risk (lr) types 6, 11, 32, 39, 40, 42, 43, 44, 54, 55, 57, 61, 71, 72, 81, 83, 84 and 86, and 'possible high-risk' types 26, 30, 34, 53, 67, 69, 70, 73, 82, and 85, was performed.(19, 20)

Statistical analysis

Cumulative VSCC incidence was determined in three subgroups: HPV-associated HSIL, p53 mutant HPV-independent VIN (HPV-independent VIN/p53mut) and p53 wild-type HPV-independent VIN (HPV-independent/p53wt). Additionally, stratified analyses for morphological subtype of HPV-independent VIN, i.e. differentiated or non-differentiated ('HPV-associated-like') were done. Cumulative VSCC incidence was calculated from baseline hg-VIN to the date of VSCC with the Kaplan Meier adjusting for censoring with 95% confidence interval (CI).(6) Details on censoring are described in Supplementary File 1.(21) Cancer risk differences were evaluated by log-rank tests. The level of statistical significance was set at 0.05. Statistical analysis was performed using IBM SPSS Statistics software for Windows version 28.0 (IBM Corporation, Armonk, NY) and graphs were produced in GraphPad Prism 9.

Results

Study population

From 791/894 (88.5%) patients, tissue blocks of baseline hg-VIN were retrieved. Subsequently, 40 cases were excluded due to insufficient tissue, resulting in 751 (84.0%) vulvar lesions. Median follow-up time was 17.3 years (range 8.3-35.4).

Categorization of HPV-associated and HPV-independent lesions

Final categorization in relation to the original diagnoses and age is shown in Table 1. Most lesions were HPV-associated (88.4%) and were categorized as HSIL (77.0%) or LSIL (10.9%). Three cases (0.4%) had presence of micro-invasive disease and were categorized as VSCC. A minority of lesions (10.9%) was HPV-independent and categorized as HPV-independent VIN (6.1%), non-dysplastic lesions (4.7%), or VSCC (0.1%). The diagnosis was inconclusive for 1.1% of lesions, mainly because no distinction between HPV-associated and HPV-independent could be made. Patients with HPV-associated lesions had a lower median age compared to patients with HPV-independent lesions ($p<0.001$) but a wide range was observed.

Categorization in relation to the IHC and HPV genotyping results is depicted in Table 2, Table 3, and Supplementary File 2.

Table 1. Categorisation of vulvar lesions after reassessment in relation to the original diagnosis and age at baseline

Final categorization	**Original diagnosis**				**Age, years**	
	HSIL	**DVIN**	**Total**	**(%)**	**Median**	**(range)**
	743	**8**	**751**	**(100)**	**45.0**	**(16-92)**
HPV-associated	663	1	663	(88.4)	44.0	(17-91)
HSIL	578	0	578	(77.0)	45.0	(17-90)
LSIL	81	1	82	(10.9)	39.0	(19-91)
VSCC	3	0	3	(0.4)	39.0	(37-48)
HPV-independent	75	7	82	(10.9)	67.0	(16-92)
HPV-independent VIN	39	7	46	(6.1)	72.0	(35-92)
Non-dysplastic	35	0	35	(4.7)	58.0	(16-79)
VSCC	1	0	1	(0.1)	67.0	NA
Inconclusive	6	0	6	(0.8)	75.0	(46-88)

HPV: human papillomavirus; dVIN: differentiated vulvar intraepithelial neoplasia; HSIL: high-grade squamous intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; VSCC: vulvar squamous cell carcinoma; NA: not applicable.

Table 2. Immunohistochemical staining patterns of p16^{INK4a}, p53 and ki-67 in human papillomavirus (HPV)-associated and HPV-independent vulvar lesions

			HPV-associated				**HPV-independent**			
			HSIL+		**LSIL**		**HPV-independent VIN+**		**Non-dysplastic**	
P16^{INK4a}	Negative	Negative	1	(0.2)	23	(28.0)	32	(68.1)	26	(74.3)
		Patchy	5	(0.9)	34	(41.5)	14	(29.8)	9	(25.7)
	Block positive	≤1/3	50	(8.6)	15	(18.3)	0	(0)	0	(0)
		≤2/3	289	(49.8)	10	(12.2)	0	(0)	0	(0)
		>2/3	235	(40.5)	0	(0)	1	(2.1)	0	(0)
P53	Wild-type	Scattered	278	(48.0)	61	(74.4)	16	(34.8)	35	(100)
		Mid-epithelial	300	(51.8)	21	(25.6)	0	(0)	0	(0)
	Mutant	Positive	1	(0.2)	0	(0)	19	(41.3)	0	(0)
		Null	0	(0)	0	(0)	11	(23.9)	0	(0)
		Cytoplasmic	0	(0)	0	(0)	0	(0)	0	(0)
Ki-67	Not increased	Normal	2	(0.3)	20	(24.4)	6	(12.8)	19	(54.3)
		'Viral' ki-67	38	(6.6)	32	(39.0)	0	(0)	0	(0)
	Increased	≤1/3	36	(6.2)	55	(67.1)	36	(76.6)	14	(40.0)
		≤2/3	317	(54.7)	7	(8.5)	4	(8.5)	2	(5.7)
		>2/3	224	(38.7)	0	(0)	1	(2.1)	0	(0)

Note: in four lesions one or more stains could not be assessed (1x p16^{INK4a}, 4x p53 and 2x ki-67).

HSIL+: high-grade squamous intraepithelial lesion, including three HPV-associated vulvar squamous cell carcinomas; LSIL: low-grade squamous intraepithelial lesion; HPV-independent VIN+: HPV-independent vulvar intraepithelial neoplasia, including one HPV-independent vulvar squamous cell carcinoma.

Table 3. High-risk and low-risk human papillomavirus (HPV) genotype distribution per disease category. Type-specific positivity includes those contributed by multiple infections

	HPV-associated				HPV-independent			
	HSIL+		LSIL		HPV-independent VIN+		Non-dysplastic	
Overall HPV positive	557/559	(99.6)	56/62	(90.3)	5/34	(14.7)	0/11	(0)
High-risk HPV positive	553	(99.3)	43	(76.8)	4	(80.0)	0	(0.0)
Single high-risk HPV type	535	(96.1)	42	(75.0)	4	(80.0)	0	(0.0)
Multiple high-risk HPV types	18	(3.2)	1	(1.8)	0	(0.0)	0	(0.0)
High-risk HPV genotype 16/18	479	(86.0)	32	(57.1)	3	(60.0)	0	(0.0)
Type 16	453	(81.3)	30	(53.6)	3	(60.0)	0	(0.0)
Type 18	27	(4.8)	3	(5.4)	0	(0.0)	0	(0.0)
High-risk HPV genotype non-16/18	89	(16.0)	11	(19.6)	1	(20.0)	0	(0.0)
Type 31	2	(0.4)	0	(0.0)	0	(0.0)	0	(0.0)
Type 33	41	(7.4)	2	(3.6)	0	(0.0)	0	(0.0)
Type 35	1	(0.2)	1	(1.8)	0	(0.0)	0	(0.0)
Type 45	5	(0.9)	0	(0.0)	0	(0.0)	0	(0.0)
Type 51	4	(0.7)	1	(1.8)	0	(0.0)	0	(0.0)
Type 52	1	(0.2)	1	(1.8)	0	(0.0)	0	(0.0)
Type 56	2	(0.4)	1	(1.8)	0	(0.0)	0	(0.0)
Type 59	2	(0.4)	0	(0.0)	0	(0.0)	0	(0.0)
Type 66*	2	(0.4)	0	(0.0)	0	(0.0)	0	(0.0)
Type undetermined (variant X)	9	(1.6)	0	(0.0)	0	(0.0)	0	(0.0)
Type non-16/18, not further specified**	21	(3.8)	5	(8.9)	1	(20.0)	0	(0.0)
Tested for additional HPV types	8/559	(1.4)	16/62	(25.8)	15/34	(44.1)	2/11	(18.2)
Low-risk HPV positive	4	(0.7)	13	(23.2)	1	(20.0)	0	(0)
Single low-risk HPV type	3	(0.5)	12	(21.4)	1	(20.0)	0	(0)
Multiple low-risk HPV types	1	(0.2)	1	(1.8)	0	(0)	0	(0)
Low-risk HPV genotype								
Type 6	2	(0.4)	11	(19.6)	0	(0)	0	(0)
Type 11	0	(0.0)	0	(0.0)	1	(20.0)	0	(0)
Type 26*	1	(0.2)	0	(0.0)	0	(0)	0	(0)
Type 34*	1	(0.2)	0	(0.0)	0	(0)	0	(0)
Type 42	0	(0.0)	3	(5.4)	0	(0)	0	(0)
Type 83	1	(0.2)	0	(0.0)	0	(0)	0	(0)

HSIL+: high-grade squamous intraepithelial lesion, including three HPV-associated vulvar squamous cell carcinomas; LSIL: low-grade squamous intraepithelial lesion; HPV-independent VIN+: HPV-independent VIN, including one HPV-independent vulvar squamous cell carcinoma.

* IARC (International Agency for Research on Cancer) Group 2b ('possibly carcinogenic').(19)

** 'High-risk HPV Type non-16/18, not further specified' was used for cases that could not be subtyped due to insufficient DNA.

HPV-associated high-grade squamous intraepithelial lesions (HSIL)

HSIL was usually easy to diagnose by hematoxylin and eosin (H&E) assessment only (Figure 2, A-D). In HSIL, block-positive $p16^{INK4a}$ was observed in 99.0%, hr-HPV was detected in 99.3% of HPV positive HSIL, and HPV16 was present in 81.3% of HPV positive HSIL. Six (1.0%) HSIL were $p16^{INK4a}$ negative (Figure 2, E-H), all positive for HPV16, with wild-type p53 staining and with HSIL morphology, supported by increased ki-67 in ≥2/3 of the epithelium. Six HSIL were negative for hr-HPV, all block-positive for $p16^{INK4a}$, with wild-type p53 staining and 4/6 positive for lr-HPV or 'possible hr'-HPV (type 6, 6/34, 26 and 83). Increased ki-67 in ≥2/3rd of the epithelium was encountered in 93.6% of HSIL. Many HSIL showed reduced staining intensity of p53, mimicking a p53 mutant null pattern (Figure 3, A-D). P53 mid-epithelial staining with sparing of the basal cell layer was observed in 51.6% of HSIL (Figure 3, E-H). One (0.2%) HSIL showed mutant positive p53 staining in 30% of the lesion. This lesion showed obvious HSIL morphology, block-positive $p16^{INK4a}$, and harboured hr-HPV.

HPV-associated low-grade squamous intraepithelial lesions (LSIL)

Examples of LSIL are shown in Figure 4, A-D. In LSIL, $p16^{INK4a}$ block-positivity was found in 30.5%. Viral lesions without dysplasia (n=18) were all $p16^{INK4a}$ negative. Overall HPV and hr-HPV were detected in 90.3% and 76.8%, respectively. HPV16 was detected in 53.6% of HPV positive LSIL. Of 19 hr-HPV negative LSIL, 86.7% (13/15 tested) were lr-HPV positive. Of all LSIL, 39.0% demonstrated a 'viral' ki-67 (Figure 4, A-D). P53 mid-epithelial staining was seen in 25.6% of LSIL, both in $p16^{INK4a}$ positive and negative lesions (Figure 4, E-H).

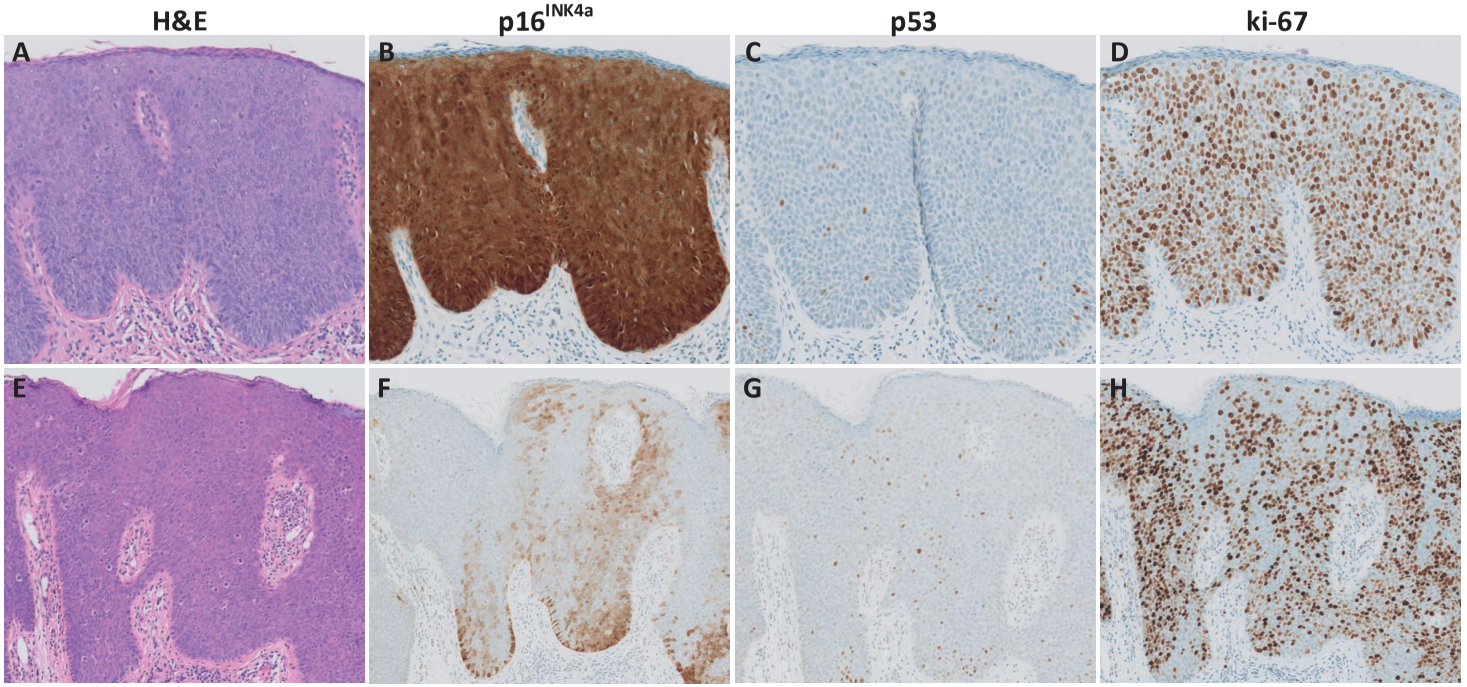


Figure 2. HPV-associated vulvar high-grade squamous intraepithelial lesion (HSIL) (A-D) Representative example of 'classical' HSIL with block-positive p16^{INK4a}, wild-type, scattered p53 staining and full-thickness increased ki-67. (E-H) HSIL with patchy (negative)p16^{INK4a}, HSIL morphology, wild-type, scattered p53 and full-thickness increased ki-67.

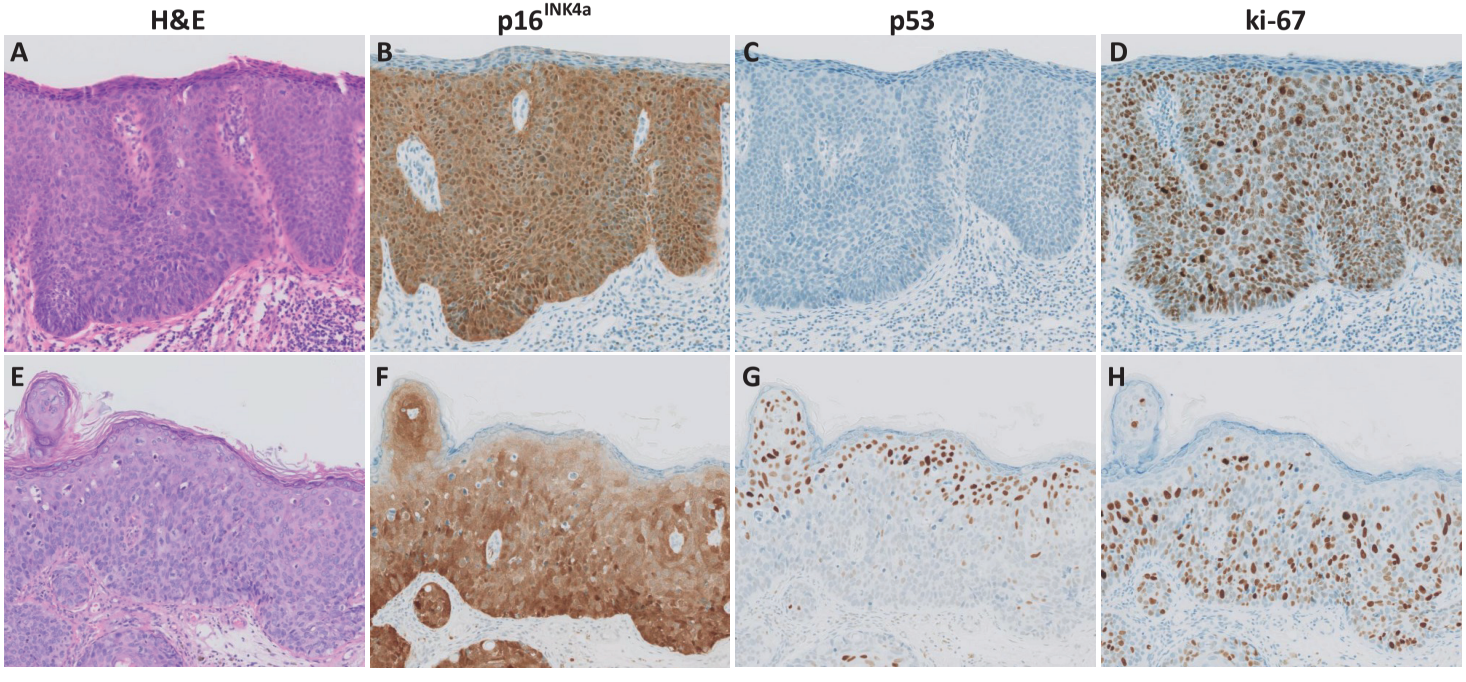


Figure 3. HPV-associated vulvar high-grade squamous intraepithelial lesion (HSIL (A-D) HSIL with wild-type, reduced p53 staining, mimicking a mutant null pattern. (E-H) HSIL with wild-type p53 mid-epithelial staining with sparing of the basal cell layer, which can mimic mutant positive staining, and with positive $p16^{INK4a}$.

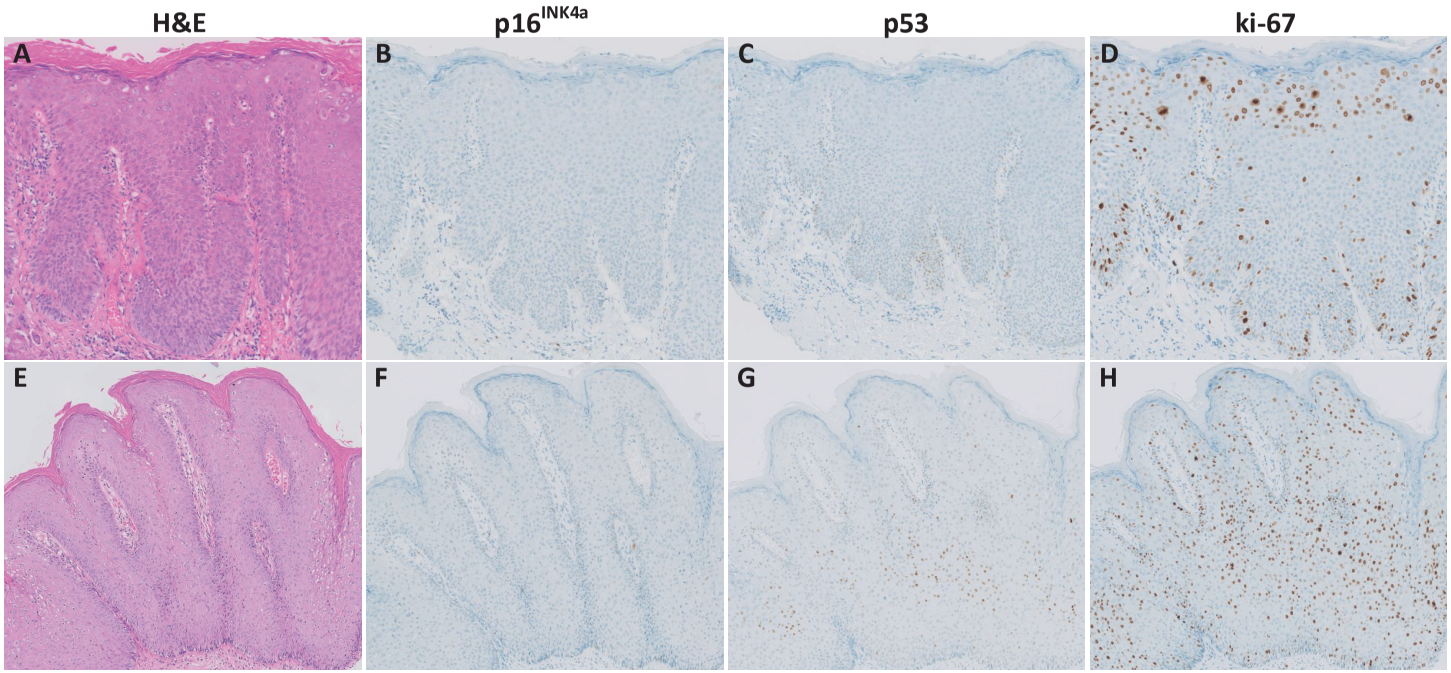


Figure 4. HPV-associated vulvar low-grade squamous intraepithelial lesion (LSIL) (A-D) LSIL with 'viral' ki-67 in scattered individual koilocytic cells in the upper epithelium with lesser staining in the lower epithelium, mimicking transepithelial increased ki-67. (E-H) LSIL with wild-type p53 mid-epithelial staining with sparing of the basal cell layer and negative $p16^{INK4a}$.

HPV-independent VIN

Of 46 HPV-independent VIN, 39 (84.8%) had originally been reported as HSIL. Mutant p53 staining was present in 65.2%: 41.3% with positive staining and 23.9% with null staining (Figure 5, A-D. The remainder 34.8% had wild-type, scattered p53 staining (Figure 5, E-H). P53 mid-epithelial staining was not observed. In five HPV-independent VIN, HPV was detected (four hr-HPV and one lr-HPV), all in combination with mutant p53 and negative $p16^{INK4a}$. Morphology was heterogeneuos, with non-differentiated ('HPV-associated-like') morphology in 41.3% (Figure 6, A-H). Of those, 89.5% had mutant p53 staining, in 94.7% in combination with negative $p16^{INK4a}$ staining and in 90.9% (10/11) of tested cases without hr-HPV. One HPV-independent VIN with non-differentiated ('HPV-associated-like') morphology showed mutant p53 staining in combination with block-positive $p16^{INK4a}$ and negative HPV (Figure 6, E-H).

Non-dysplastic lesions

Non-dysplastic, non-viral lesions exclusively showed negative $p16^{INK4a}$, negative HPV, and scattered wild-type p53 staining. Ki-67 was increased in ≤1/3 of the epithelium in 40.0% and in ≤2/3 of the epithelium in 5.7%. Non-dysplastic lesions included LS (17.1%), inflammation (31.4%), reactive changes (31.4%), (fibro-)epithelial polyps (5.7%), and no abnormalities (14.3%).

Vulvar cancer risk in patients with hg-VIN

Four patients (0.5%) with microinvasive disease at histopathological reassessment were excluded from VSCC analyses. In HPV-associated HSIL, the 10-year cancer incidence was 8.0% (Table 4, Figure 7A). HSIL with vulvar carcinoma in follow-up tested for HPV (n=59) harboured HPV16 in 86.4%, HPV18 in 3.4%, HPV18/hr-HPV non-16/18 undetermined ('variant X') in 1.7%, and HPV33 in 3.4%. In 6.8%, hr-HPV non-16/18 type was not further specified. The prevalence of HPV genotypes did not significantly differ between HSIL with or without VSCC in follow-up.

In HPV-independent VIN, the 10-year cancer incidence was 53.6%, 67.4% for HPV-independent VIN/p53mut and 27.8% for HPV-independent VIN/p53wt (p=0.004). The 10-year cancer incidence in HPV-independent VIN with non-differentiated ('HPV-associated-like') versus differentiated morphology was 73.7% versus 39.3% (p=0.001), Figure 7B. Median time to cancer was significantly shorter for HPV-independent VIN compared to HSIL: 1.8 versus 6.0 years (p<0.001), for p53 mutant versus wild-type IHC: 1.5 versus 5.1 years (p=0.010), and for non-differentiated ('HPV-associated-like') versus differentiated morphology: 0.5 versus 3.2 years (p=0.002).

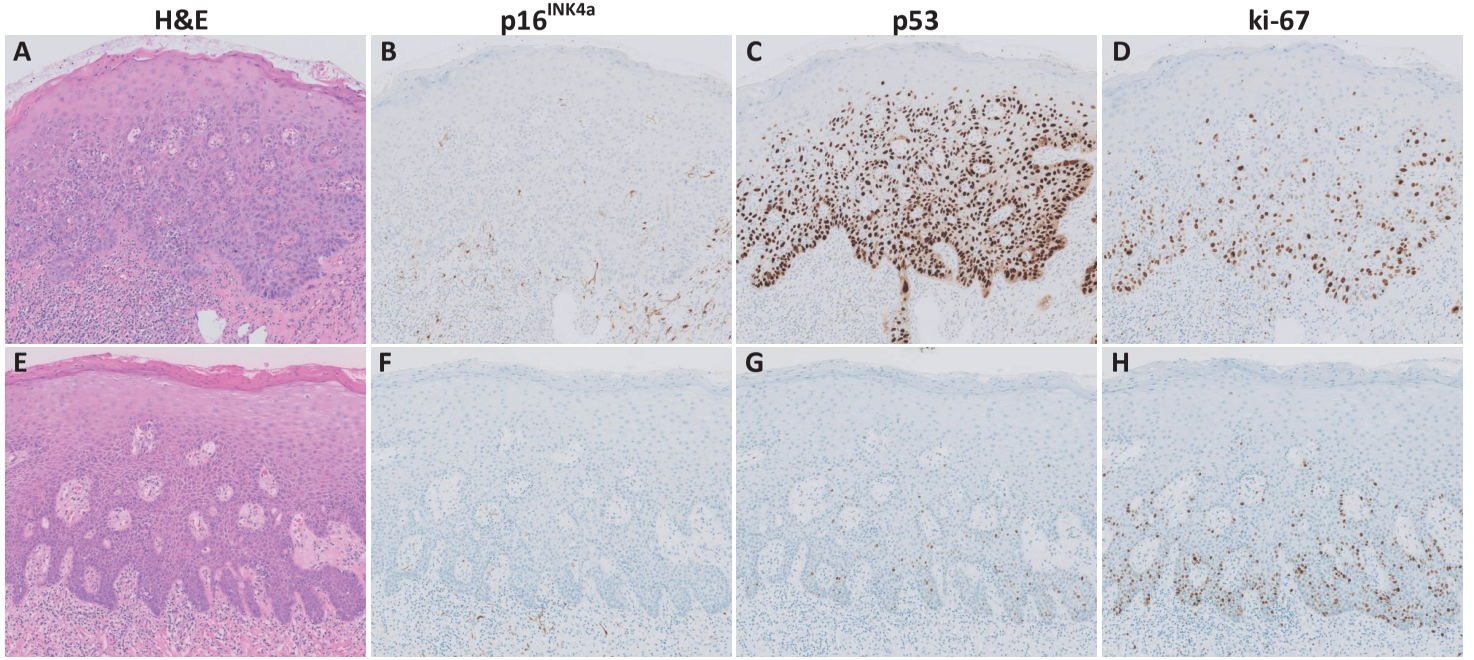


Figure 5. HPV-independent vulvar intraepithelial neoplasia (VIN) (A-H) Representative examples of HPV-independent VIN with differentiated morphology, negative $p16^{INK4a}$ and respectively mutant (C) versus wild-type scattered p53 staining (G).

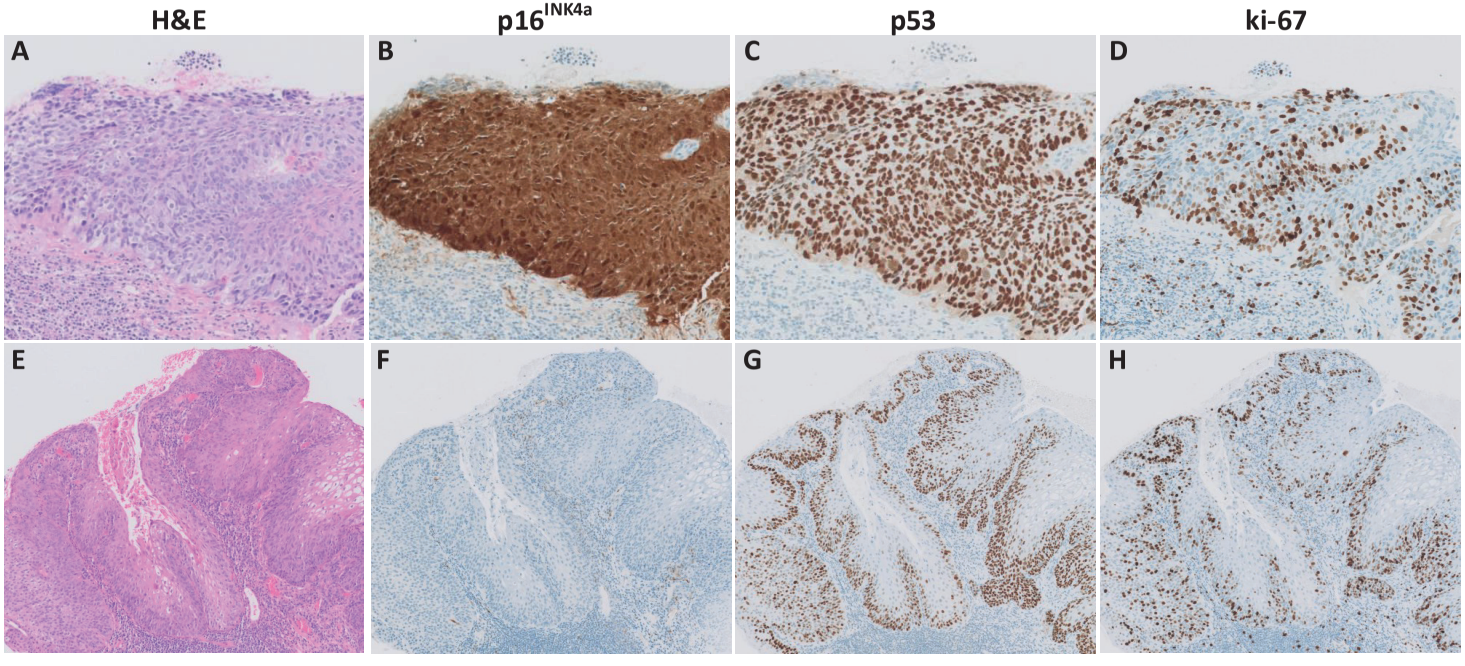
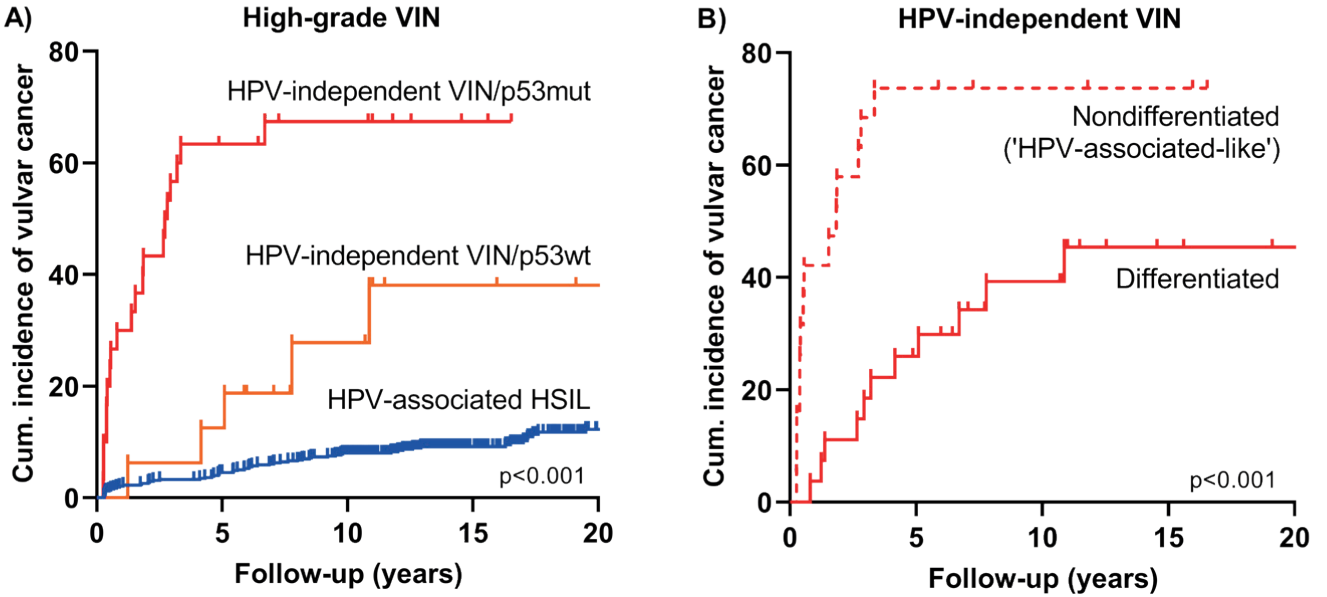


Figure 6. HPV-independent vulvar intraepithelial neoplasia (VIN) with non-differentiated ('HPV-associated-like') morphology (A-H) Both cases had negative HPV DNA and mutant positive p53 staining. In (A-D) basaloid morphology is seen, whereas in (E-H) wide and deep rete ridges, with moderate pleomorphism and koilocytic-like changes are seen.



HPV-ind/p53mut	30	10	7	2	0
HPV-ind/p53wt	16	14	8	4	2
HPV-ass HSIL	578	548	496	303	174

Nondiff.	19	5	3	2	0
Diff.	27	19	12	4	2

Figure 7. Cumulative incidence of vulvar cancer in high-grade VIN. A) stratified for three subtypes of high-grade VIN: HPV-associated HSIL, HPV-independent VIN/p53 mutant, and HPV-independent VIN/p53 wild-type. B) HPV-independent VIN with differentiated morphology versus non-differentiated ('HPV-associated-like') morphology.

HPV-independent VIN: human papillomavirus-independent vulvar intraepithelial neoplasia; HSIL: high-grade squamous intraepithelial lesion.

Table 4. Risk of vulvar squamous cell carcinoma (VSCC), including time to VSCC, per disease category

	Absolute VSCC risk		**Cumulative incidence of VSCC (95% confidence interval)**						**Median time to VSCC, years (range)**	
	No.	**%**	**1 year**		**5 years**		**10 years**			
HPV-associated HSIL	61/578	10.6	2.1	(0.9-3.3)	4.5	(2.7-6.3)	8.0	(5.8-10.2)	6.0	(0.3-24.2)
HPV-independent VIN	25/46	54.3	19.6	(8.2-31.0)	45.7	(31.4-60.0)	53.6	(38.7-68.5)	1.8	(0.3-10.9)
P53 mutant	20/30	66.7	30.0	(13.5-46.5)	63.3	(46.1-80.5)	67.4	(50.3-84.5)	1.5	(0.3-6.7)
P53 wild-type	5/16	31.3	0.0	NA	12.5	(0.0-28.8)	27.8	(3.9-51.7)	5.1	(1.2-10.9)
Differentiated	11/27	40.7	3.7	(0.0-10.8)	25.9	(9.4-42.4)	39.3	(19.9-58.7)	3.2	(0.8-23.3)
Non-differentiated	14/19	73.7	42.1	(20.0-64.2)	73.7	(53.9-93.5)	73.7	(53.9-93.5)	0.5	(0.3-16.5)

HSIL: high-grade squamous intraepithelial lesion; HPV-independent VIN: human papillomavirus-independent vulvar intraepithelial neoplasia; NA: not applicable.

Discussion

Our study on vulvar lesions of 751 patients, all originally reported as hg-VIN, demonstrated that immunohistochemical markers p16^{INK4a} and p53 are very valuable for adequate categorization into HPV-associated- and HPV-independent types. Given the broad morphologic spectrum of HPV-independent VIN, including the morphological overlap with HPV-associated SIL, original categorization based on H&E staining without the use of IHC led to an inadequate diagnosis of 84.8% of HPV-independent VIN. Typing of VIN is of utmost importance given the different 10-year cancer risk of 8.0% for HPV-associated HSIL and 53.4% for HPV-independent VIN. Strikingly, HPV-independent VIN/p53mut had a twice as high 10-year cancer risk compared to HPV-independent VIN/p53wt (67.4% versus 27.8%). In addition, HPV-independent VIN with non-differentiated ('HPV-associated-like') morphology had the highest 10-year cancer risk of 73.7%, compared to a 39.3% risk with differentiated morphology ($p<0.001$). Besides the higher cancer risk, both p53 mutant and non-differentiated subgroups of HPV-independent VIN had a much shorter time to cancer progression.

HPV-associated vulvar lesions

Consistent with the literature, 99.0% of HSIL were p16^{INK4a} block-positive and 98.9% harboured hr-HPV, mostly HPV16.(22) Among HSIL progressing to vulvar cancer, 86.4% had HPV16, which was not statistically different from HSIL without progression, possibly because the vast majority of HSIL were HPV16 positive.

In LSIL we observed p16^{INK4a} in 30.5% and hr-HPV positivity in 76.8%, which is both higher compared to the literature, reporting rates of respectively 4-20% and 10-42%.(23-26) A likely explanation is that the LSIL in our study comprised a selected series, all originally diagnosed as hg-VIN. Characteristic of LSIL in our series was the high proportion (39.0%) of 'viral' ki-67 staining with increased numbers of positive cells in the upper epithelial layers compared to the lower epithelial layers. A 'viral' ki-67 staining pattern was not observed in HPV-independent VIN and therefore it can aid in distinguishing it from LSIL, as HPV-independent VIN and LSIL may show morphologically overlapping features with negative p16^{INK4a} and wild-type p53. To our best knowledge, 'viral' ki-67 staining has only been described once before, in 2/11 HPV-positive vulvar seborrheic keratoses.(27) It is important to recognize a 'viral' ki-67 pattern, as it may erroneously lead to upgrading and overtreatment of vulvar lesions.

Both wild-type p53 staining with reduced intensity and mid-epithelial patterns were also exclusively seen in HPV-associated SIL, should not be confused with true mutant patterns as seen in HPV-independent VIN.(9, 17, 28, 29) While *TP53* mutations can occur in HPV-associated SIL, they are usually non-functional.(30, 31) These cases show combined $p16^{INK4a}$ positive/p53 wild-type patterns indicating that hr-HPV drives the pathogenesis in these lesions. Reduced p53 in HPV-associated SIL is likely explained by p53 degradation by the E6 protein of oncogenic HPV.(32, 33) P53 mid-epithelial staining is not fully understood and has been described for vulvar, anal and cervical precursors.(31, 34, 35) One likely explanation is that an E6 splice variant is expressed which cannot degrade p53.(36, 37)

HPV-independent vulvar lesions

The diagnosis of HPV-independent VIN is challenging, as shown by multiple reports in the recent years.(8, 13, 38-40) The histomorphology of HPV-independent VIN displays a broad spectrum, from characteristic HPV-independent VIN to more subtle changes of precancerous tissue. Molecular aberrations can extend beyond epithelium with only deceptively minimal cytologic atypia, as recently described.(38, 41)

The frequency of mutant p53 staining in HPV-independent VIN in our series was 65.2%, which is in line with other series, describing rates from 42 to 100%.(11, 42) Interestingly, HPV-independent VIN/p53mut had a significant higher cancer risk compared to HPV-independent VIN/p53wt. In addition, 41.3% of HPV-independent VIN in our series had non-differentiated ('HPV-associated-like') morphology, a lesion type first described in 2009 and histologically indistinguishable from HPV-associated SIL.(14) This subset of HPV-independent VIN had the highest 10-year cancer risk (73.7%) and the shortest median time to carcinoma (0.5 years). Possible explanations are the high rate of mutant p53 IHC, or the basaloid histology, which is associated with worse prognosis in other carcinoma types, especially in SCC of the head and neck.(43) Our results are consistent with the recent recognition that VSCCs with mutant p53 or non-differentiated ('HPV-associated-like') morphology exhibit higher recurrence rates and poorer survival than their counterparts.(39, 42, 44) All these observations highlight the importance of using biomarkers $p16^{INK4a}$ and p53 IHC for VIN typing.

In addition to dVIN, the 2020 WHO classification of female genital tumours has included two HPV-independent VIN/p53wt lesions: differentiated exophytic vulvar intra-epithelial lesion (DEVIL) and vulvar acanthosis with altered differentiation (VAAD).(1) DEVIL is defined by an exophytic growth pattern and absence of significant nuclear atypia.(45, 46) In our study, most HPV-independent VIN/p53wt were not

exophytic, a few showed marked atypia and some showed non-differentiated morphology. Therefore, the term 'HPV-independent VIN/p53wt' probably better delineates the disease than former terms VAAD, DEVIL, VAM (vulvar aberrant maturation) and vaVIN (HPV-independent VIN/p53wt verruciform acanthotic VIN).(47) It should be emphasized that VAAD was not encountered in our series because those lesions have originally not been reported as hg-VIN. HPV-independent VIN/p53wt precursors likely have a broader morphological spectrum than currently described. Verrucous lichen simplex chronicus carries a relatively high cancer risk, but is often still regarded as reactive instead of a premalignant lesion. Given the morphologic overlap with reactive lesions, objective biomarkers are needed to identify HPV-independent VIN/p53wt vulvar lesions with a high cancer risk. DNA methylation has shown promising results with an 87% detection rate in HPV-independent VIN.(48, 49) Alternatively, CK17 and SOX2 immunohistochemistry showed higher expression in HPV-independent VIN compared to non-dysplastic vulvar tissues, but more studies are needed.(50, 51)

HPV DNA testing is useful in some cases, but one should be aware of the pitfalls. Mere detection of HPV DNA or positive $p16^{INK4a}$ alone does not prove a functional role of HPV. In our series, one HPV-independent VIN had mutant positive p53 and positive $p16^{INK4a}$ IHC, and was classified as HPV-independent VIN given the negative HPV DNA. Positive $p16^{INK4a}$ in this case was not caused by hr-HPV, but possibly by a mutation in *CDKN2A*.(30) Hr-HPV was detected in 10.9% of HPV-independent VIN, all with mutant p53 staining and negative $p16^{INK4a}$. Two other studies have shown comparable high numbers, of 6.4% and 12.5%.(42, 52) A possible explanation for the high HPV prevalence in HPV-independent VIN is the presence of LS, in which defective viral clearance or reactivation of a latent HPV infection can occur, because of prolonged use of topical corticosteroids.(53)

We acknowledge several limitations of our study. Given the retrospective nature of this study, clinical information was limited and was not used for categorization of the lesions. Besides adaptations in international classification systems, both the use of IHC and the awareness of HPV-independent VIN increased during the study period (1991-2011), limiting direct comparison of the initial pathology report to current practice.(1, 54) Also, we have not been able to confirm p53 IHC with p53 mutational status, although the concordance is known to be high (91-97%).(16, 17, 55)

Our study also has several strengths. To the best of our knowledge, this is the largest study that has comprehensively characterized vulvar lesions, originally diagnosed as hg-VIN with respect to IHC of $p16^{INK4a}$, p53 and ki-67, including HPV genotyping and

long-term vulvar cancer risk. Selection of our cohort was population-based instead of institutional based. Correlations between morphology, HPV genotype and vulvar cancer risk have not been established before in hg-VIN. We have used a standardized and clinically validated methodology to detect HPV DNA, allowing our results to provide valuable data on the expected effect of vaccination in The Netherlands.

Conclusion

We were the first to demonstrate in a large population-based series that HPV-independent VIN with p53 mutant IHC or non-differentiated ('HPV-associated-like') morphology has distinctive pathological and behavioural features. Both subtypes are highly aggressive and warrant closer surveillance after surgery. In order to allow correct typing of hg-VIN, performance of $p16^{INK4a}$ and p53 IHC on at least each newly diagnosed VIN lesion is highly recommended. Future work should focus on clinicopathological and molecular factors searching for additional biomarkers, which are necessary for accurate diagnosis of HPV-independent VIN and for cancer risk stratification of HPV-associated SIL.

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Supplementary File 1

Details of tissue processing, immunohistochemistry of p16^{INK4a}, p53 and ki-67, DNA isolation and HPV DNA testing.

Tissue processing

FFPE tissue block sectioning was performed according to the sandwhich method. The first and last sections were used for hematoxylin and eosin (H&E) staining to ensure the presence of lesional tissue, and in-between sections were collected in sterile PCR tubes for DNA isolation.

Immunohistochemistry

The Optiview detection kit with the automated 100 BenchMark ULTRA IHC/ISH system (Roche) was used to perform immunostaining of p16^{INK4a}, p53 and ki-67. For immunostaining of respectively p16^{INK4a}, ki-67 and p53 were mouse monoclonal antibodies against the p16^{INK4a} antigen (clone E6H4; Roche, Basel, Switzerland), the ki-67 antigen (clone MIB-1; Dako, Glostrup, Denmark) and the p53 antigen (clone DO-7; Roche, Basel, Switzerland) used.

P16^{INK4a} was scored based on diffuse or 'block' staining of the cell cytoplasm and/or nucleus in the epithelium. P53 was scored based on the localization and intensity of the immunoreactivity within the nuclei or cytoplasm of the epithelium. Ki-67 was scored based on the localization and extent of the immunoreactivity within the nuclei of the epithelium. Further categorization of p16^{INK4a}, p53 and ki-67 staining is described in the Materials and Methods section.

DNA isolation

DNA was isolated using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was eluted with the easyMAG 3 elution buffer (bioMérieux, Boxtel, the Netherlands). DNA concentration was measured using Qubit (Thermo Fisher Scientific Inc, Qiagen).

Human papillomavirus (HPV) genotyping

High-risk (hr-)HPV DNA testing was performed using the QIAscreen HPV PCR test (QIAgen, Hilden, Germany), according to manufacturer's instructions. The assay is directed against the HPV E7 region and detects hr-HPV type 16 and 18, as well as 13 'other' hr-HPV genotypes (i.e. 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) as a pool.1 Samples with HPV type 'other' were further subtyped with a L1-region-based GP5+6+ PCR, followed by a microsphere bead-based assay (Luminex xMAP; Luminex

Corp, Austin, TX, USA). When no hr-HPV type 'other' could be assigned, hr-HPV was labelled 'HPV undetermined ('variant X')'. When no sufficient DNA was available to further subtype hr-HPV type 'other', the hr-HPV result was labelled 'non-16/18 type, no further specified)'. Beta-globin was used as an internal quality control for each sample. A cycle threshold (Ct) of >30 for beta-globin was considered invalid when no HPV was found.

To detect presence of additional HPV types, the L1-region-based GP 5+6+ PCR was used followed by enzymatic immunoassay (EIA).[2] Samples with a positive EIA result were subjected to a reverse line blot genotyping assay. This assay included low-risk types 6, 11, 32, 39, 40, 42, 43, 44, 54, 55, 57, 61, 71, 72, 81, 83, 84, 86, including 'possible high-risk types' 26, 30, 34, 53, 67, 69, 70, 73, 82, and 85.[3]

Statistical analysis of vulvar squamous cell carcinoma (VSCC) risk

Follow-up time was calculated from the first date of the high-grade VIN histological diagnosis to the first date of the VSCC histological diagnosis, as described earlier.[4] Patients who did not develop VSCC had an end date set equal to the earliest date of either their expected date of death or the date of data extraction from the nationwide histopathology and cytopathology data network and archive (PALGA). The expected date of death was retrieved from age-dependent life expectancy tables of Statistics Netherlands at the time of the last vulvar pathology report.[5]

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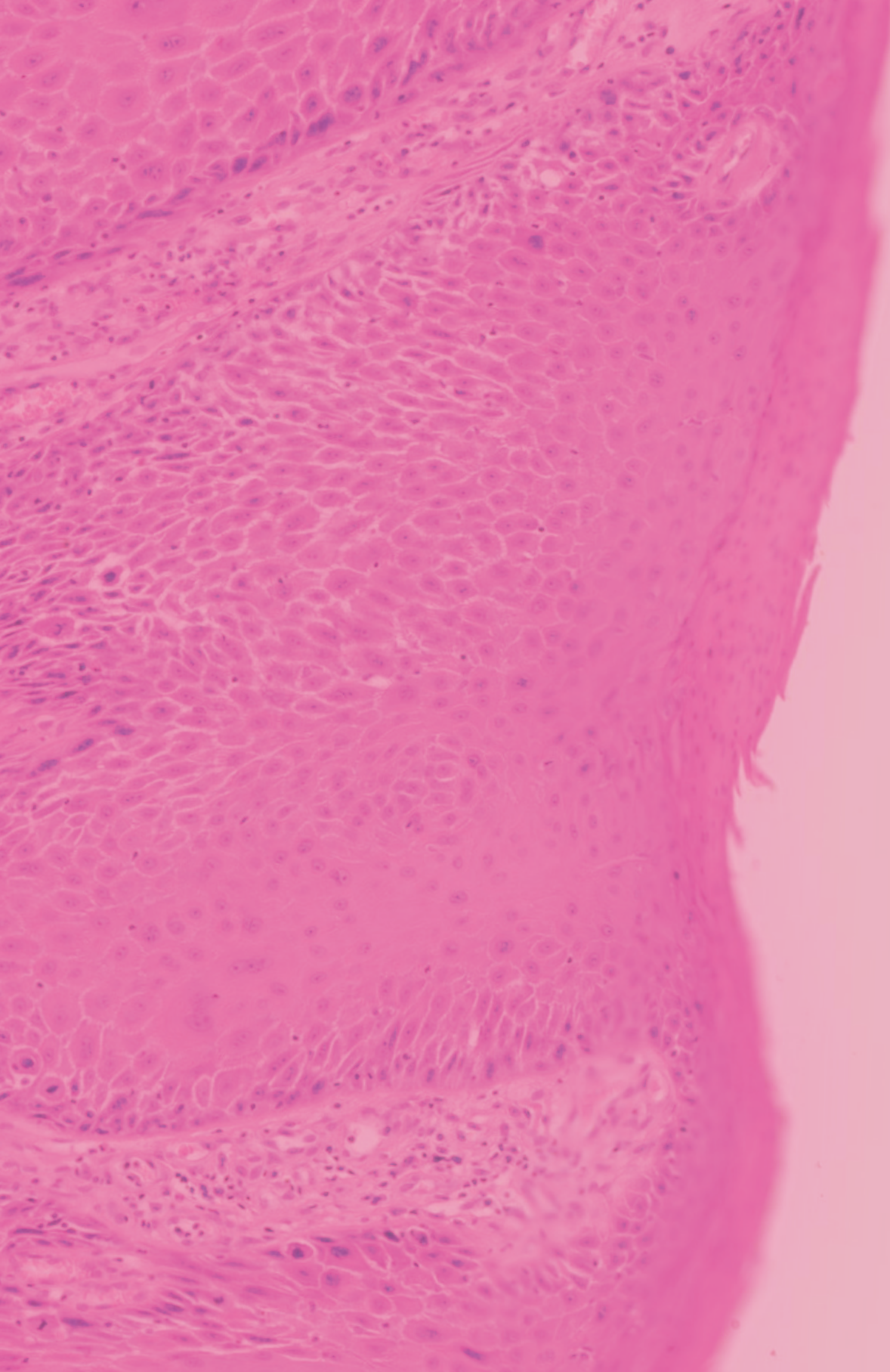
Supplementary File 2

Table overview of reaching final classification of 741 originally diagnosed high-grade VIN

Morphology	No.	P16^{INK4a}	P53	HPV	Remarks
HPV-associated HSIL (n=578)					
HSIL	555	Pos	Wt	Pos/Neg	Classical case
DD: HSIL vs LSIL	12	Pos	Wt	Pos/Neg	Positive p16 decisive; ki-67 ≥2/3rd (n=9)
HSIL	6	Neg	Wt	Pos	Combination wild-type p53, ki-67 ≥2/3rd and positive hr-HPV decisive
DD: HSIL vs HPV-ind. VIN	5	Pos	Wt	Pos/Neg	Combination positive p16 and wild-type p53 decisive
HPV-associated LSIL (n=82)					
LSIL	65	Pos/Neg	Wt	Pos/Neg	Classical case
DD: LSIL vs HSIL	10	Neg	Wt	Pos/Neg	Negative p16 decisive; ki-67 ≤1/3rd (n=9)
DD: LSIL vs HPV-ind. VIN	4	Pos/Neg	Wt	Pos/Neg	Combination wild-type p53 with either positive p16 and/or HPV decisive; mid-epithelial (wild-type) p53 (n=2) and/or 'viral' ki-67 (n=1) support viral origin
DD: LSIL vs reactive	3	Pos/Neg	Wt	Pos/Neg	Positive p16 and/or positive HPV decisive; mid-epithelial (wild-type) p53 (n=0) and/or 'viral' ki-67 (n=1) support viral origin
HPV-independent VIN (n=46)					
HPV-independent VIN	26	Neg	Mut/Wtsc	Pos/Neg	Classical case
DD: HPV-ind. VIN vs HSIL	16	Neg	Mut	Pos/Neg	Combination mutant p53 and negative p16 decisive
DD: HPV-ind. VIN vs HSIL	2	Neg	Wtsc	Neg	Combination negative p16 and negative HPV decisive; mid-epithelial (wild-type) p53 and/or 'viral' ki-67 exclude HPV-ind. VIN
HSIL	1	Pos	Mut	Neg	Combination mutant p53 and negative HPV decisive
DD: HPV-ind. VIN vs reactive	1	Neg	Mut	Pos/Neg	Mutant p53 decisive
Non-dysplastic, non-viral (n=35)					
Non-dysplastic, non-viral	25	Neg	Wtsc	Neg	No dysplasia and no viral features
DD: reactive vs LSIL	10	Neg	Wtsc	Neg	Combination negative p16 and negative HPV; mid-epithelial (wild-type) p53 and/or 'viral' ki-67 exclude reactive lesion

DD: differential diagnosis; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; HPV-independent VIN: human papillomavirus-independent vulvar intraepithelial neoplasia; Neg: negative; Pos: positive; Wt: wild-type, scattered or mid-epithelial with basal sparing; Mut: mutant, sc: scattered.



CHAPTER 7

Clinical validation of methylation biomarkers for optimal detection of high-grade vulvar intraepithelial neoplasia

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Abstract

The precursor lesions of vulvar squamous cell carcinoma (VSCC) include human papillomavirus (HPV)-associated and HPV-independent squamous neoplasia with a varying cancer risk. This study aimed to validate the accuracy of previously identified DNA methylation markers for detection of such high-grade vulvar intraepithelial neoplasia (VIN). A large clinical series of 751 vulvar lesions, originally diagnosed as high-grade VIN, were reassessed and categorized into HPV-associated or HPV-independent vulvar disease categories. Together with 113 healthy vulvar controls, all samples were tested for 12 methylation markers with quantitative multiplex methylation-specific PCR (qMSP). Performance of individual markers and selection of an optimal marker panel for detection of high-grade VIN was determined by logistic regression analysis. *SST* was the best performing individual marker (AUC 0.90), detecting 80% of high-grade VIN cases, with excellent detection of HPV-independent VIN (95%), known to have the highest cancer risk. Merely 2% of controls tested methylation positive for *SST*. Selection of a marker panel, including *ZNF582*, *SST* and *miR124-2*, resulted in a comparably high accuracy for detection of high-grade VIN (AUC 0.89). In conclusion, we clinically validated the accuracy of 12 DNA methylation markers for detection of high-grade VIN. *SST*, as a sole marker or in a panel, provides an optimal diagnostic tool to distinguish high-grade VIN in need of treatment, particularly dVIN, from low-grade or reactive vulvar lesions. These findings warrant further prognostic validation of methylation biomarkers for cancer risk stratification of patients with VIN.

Introduction

High-grade vulvar intraepithelial neoplasia (VIN) is the precursor lesion of vulvar squamous cell carcinoma (VSCC) and is categorized as human papillomavirus (HPV)-associated vulvar high-grade squamous intraepithelial lesion (vHSIL) or HPV-independent VIN, of which the most renowned subtype is differentiated VIN (dVIN).[1, 2] VHSIL accounts for the majority of high-grade VIN lesions and is diagnosed in relatively younger patients. HPV-independent VIN usually arises in a background of lichen sclerosus (LS) or lichen planus (LP), chronic dermatoses of the anogenital area, and is mostly diagnosed in postmenopausal women.[1, 3] The incidence of both VSCC and its precursor lesions is rising.[4-6]

Patients with vHSIL and HPV-independent VIN have a varying risk of developing cancer, with a 10-year cumulative VSCC risk of 10% versus 50%, respectively.[6, 7] Due to this risk, patients with vHSIL and HPV-independent VIN frequently undergo surgical interventions resulting in physical and psychosexual morbidity.[8] However, as only a minority of vHSIL patients develop cancer, prognostic biomarkers reflecting the cancer risk could reduce the necessity for mutilating overtreatment. In contrast, HPV-independent VIN has a high cancer risk, yet is a challenging diagnosis. With regards to clinical presentation, HPV-independent VIN can resemble non-neoplastic lesions such as LS or LP. Histological characteristics can be subtle and have overlapping features mimicking reactive or inflammatory conditions such as LS, squamous hyperplasia or vulvar low-grade squamous intra-epithelial lesions (vLSIL).[2, 9, 10] As a consequence, misdiagnosis of HPV-independent VIN may occur, leading to undertreatment of patients with such potentially aggressive precursor lesions. Therefore, cancer predicting biomarkers can potentially aid in the identification of HPV-independent VIN during diagnostics.

Host cell DNA methylation is known as a hallmark in HPV-induced carcinogenesis which can lead to silencing of tumour suppressor genes.[11] In fact, DNA methylation is already appreciated as a biomarker for detection of lesions with a high cancer progression risk in cervical and anal neoplasia.[12-15] Previously we have shown that several genes showed higher methylation levels with increasing severity of disease in a well-defined cross-sectional series of vulvar lesions, including healthy vulvar tissue, high-grade VIN, high-grade VIN adjacent to VSCC and VSCC cases.[16] The purpose of the current study was to validate the accuracy of previously identified DNA methylation markers for detection of high-grade VIN in a large, revised clinical series of vulvar lesions.

Materials and Methods

Study samples and ethics

Patients diagnosed with high-grade VIN without prior or concurrent VSCC were selected from a population-based historical cohort provided by the Dutch Nationwide Pathology Databank (PALGA), as described previously.[6, 17] This historical cohort contains all vulvar pathology reports of patients diagnosed with vulvar LS, VIN and/or VSCC in the provinces Noord-Holland and Flevoland between 1991 and 2011.[18] A total of 894 patients with high-grade VIN were identified.[6] Of each patient, archived formalin-fixed paraffin-embedded (FFPE) tissue blocks of the incident high-grade VIN lesion (i.e. first biopsy with high-grade VIN diagnosis) were requested. In addition, healthy vulvar tissue samples were included as a control group. These samples were collected between 2018 and 2021 from healthy patients during aesthetic or reconstructive genital procedures in V-Klinieken, Leiden and Amsterdam UMC, location VUmc, respectively.

Sample processing

Retrieved FFPE tissue blocks were sectioned using the sandwich method, in which the first and last sections were stained with haematoxylin-eosin (H&E). In-between sections were cut for immunohistochemistry (IHC) staining. Additional in-between sections were used for DNA isolation and subsequent HPV genotyping and DNA methylation analysis. Healthy vulvar tissues were only subjected to DNA methylation analysis.

Histological reassessment and disease categories

All retrieved cases were reviewed by a gynaecopathologist (MCGB) and a senior resident in pathology (NBT). Histological reassessment included an integrated analysis of the morphology (H&E slide), p16, p53 and MIB-1 immunohistochemical staining and the HPV test result. Vulvar lesions were categorized as HPV-associated or HPV-independent lesions based on the final diagnosis after integrated analysis. Disease categories included HPV-associated vLSIL (including non-dysplastic lesions with viral changes), vHSIL or VSCC and HPV-independent non-dysplastic lesions (including LS, squamous hyperplasia and other non-specific dermatoses), HPV-independent VIN (dVIN) or VSCC. H&E slides of the control samples were assessed to confirm they comprised healthy vulvar tissue.

Immunohistochemistry methods

IHC staining was performed on all originally diagnosed high-grade VIN cases using the Ventana BenchMark Ultra immunostainer (Ventana Medical Systems, Roche, Tucson, AZ). Sections were immunostained for $p16^{INK4a}$ (E6H4 mouse monoclonal

antibody; Roche, Basel, Switzerland), p53 (DO-7 mouse monoclonal antibody to p53; Dako, Glostrup, Denmark) and MIB-1 (mouse MIB1 monoclonal antibody; Dako, Glostrup, Denmark).

DNA isolation

FFPE in-between sections were collected in 1.5ml PCR tubes. DNA isolation was performed using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA was eluted in easyMAG 3 elution buffer (bioMérieux, Boxtel, The Netherlands). Upon isolation, DNA concentrations were measured using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc, Qiagen).

HPV genotyping methods

High-risk (hr-)HPV DNA testing was performed using the QIAscreen HPV PCR test (QIAgen, Hilden, Germany), according to manufacturer's instructions. The assay is directed against the HPV E7 region and detects hr-HPV type 16 and 18, as well as 13 other hr-HPV genotypes (i.e. 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67 and 68) as a pool.[19] Low-risk (lr-)HPV DNA testing was performed for selected cases upon histological reassessment when deemed necessary to reach a final diagnosis, such as hr-HPV negative cases which had a viral morphology, HPV-independent VIN cases with a p53 wild-type pattern and inconclusive cases. To detect presence of lr-HPV, the L1-region-based GP 5+6+ PCR was used followed by enzymatic immunoassay (EIA).[20] Samples with a positive EIA result were subjected to a reverse line blot genotyping assay.

DNA methylation analysis

For methylation analysis, DNA isolates were modified by bisulphite conversion using the EZ-DNA Methylation kit (Zymo Research, Irvine, CA, USA). Quantitative methylation-specific polymerase chain reaction (qMSP) was performed on 50ng bisulphite-converted DNA samples. Four multiplex assays were tested, each targeting 3 genes and β-actin (*ACTB*) as the reference gene; *ASCL1/LHX8/ZNF582*, *GHSR/SST/ZIC1*, *CADM1/MAL/miR124-2* and *FAM19A4/PHACTR3/PRDM14*. Samples with a β-actin threshold cycle (Ct) value of >32 were regarded as invalid, as this indicated poor sample quality due to insufficient DNA or inadequate bisulphite conversion.[21] Methylation levels were normalized to the reference gene (β-actin) and calibrator of each multiplex using the comparative Ct method ($2^{-\Delta\Delta Ct}$ x 100) to obtain ΔΔCt ratios.[22]

Statistical analysis

Methylation levels were visualized for each individual marker by constructing boxplots of the log2 transformed ΔΔCt ratios for each disease category. VSCC and inconclusive cases were excluded from this analysis due to low sample sizes.

Significant differences between categories were assessed for each marker by the Kruskal-Wallis test, followed by pairwise post-hoc Mann-Whitney *U* testing with Bonferroni correction for multiple comparisons. Evaluation of the diagnostic performance of individual markers was determined by univariate logistic regression analyses, including samples with valid results for all 12 methylation markers. Additional multivariate logistic regression analysis with backward selection was performed to identify an optimal marker panel. These analyses were performed on healthy controls versus high-grade VIN cases (vHSIL and HPV-independent VIN). Predicted probabilities of each sample were obtained to construct receiver operating characteristic (ROC) curves. Diagnostic performance was quantified and assessed by the area under the curve (AUC), as well as the sensitivity and specificity at the Youden's index (*J*-)threshold. Provided that the specificity at Youden's index was at least 95%, the *J*-threshold was used to calculate the detection rate for each disease category. Univariate and multivariate models were examined for over-fitting by internal five-fold cross-validation. All analyses were performed in IBM SPSS Statistics software for Windows version 28 and graphs were produced in GraphPad Prism 9.

Results

Study population and disease categories

Of the historical cohort of 894 patients with high-grade VIN, FFPE tissue blocks were retrieved from 791 patients (88%). Subsequently, 40 cases were excluded due to insufficient residual tissue for any further processing. In addition, 113 healthy vulvar samples were collected. This resulted in a total series of 864 vulvar tissues which were included in the study.

Upon histological reassessment of the originally diagnosed high-grade VIN lesions (n=751), the majority of cases were categorized as HPV-associated vHSIL (n=575) or vLSIL (n=87) (Table 1). Most remaining cases were categorized as HPV-independent dVIN (n=44) or non-dysplastic lesions (n=31). These non-dysplastic lesions included seven cases of LS, six cases with non-specific inflammation, two cases with mild squamous hyperplasia and two (fibro-)epithelial polyps. The remaining cases (14/31) showed no abnormalities. Four cases had presence of (micro)invasion and were categorized as VSCC, of which three were HPV-associated and one was HPV-independent. Histological reassessment was inconclusive for 10 cases. Results on immunohistochemical staining of p16, p53 and MIB-1, as well as HPV genotyping, can be appreciated for each disease category in Supplementary Table 1.

Table 1. Baseline characteristics of the study population

Disease category	Total	Age
	No. (%)	Median (range)
Control cohort	113 (-)	30.0 (18 – 57)
Historical cohort, originally high-grade VIN	751 (100)	44.0 (16 – 92)
HPV-associated lesions		
vLSIL	87 (12)	39.0 (19 – 91)
vHSIL	575 (77)	45.0 (17 – 90)
VSCC	3 (<1)	39.0 (37 – 48)
HPV-independent lesions		
Non-dysplastic	31 (4)	58.0 (16 – 79)
dVIN	44 (6)	73.5 (45 – 92)
VSCC	1 (<1)	67.0 (67 – 67)
Inconclusive	10 (1)	67.5 (35 – 88)

Abbreviations: dVIN, differentiated vulvar intraepithelial neoplasia; HPV, human papillomavirus; vLSIL, vulvar low-grade squamous intraepithelial lesion; vHSIL, vulvar high-grade squamous intraepithelial lesion; VSCC, vulvar squamous cell carcinoma.

DNA methylation levels across vulvar disease categories

During tissue processing, 20 cases had insufficient FFPE-material for DNA isolation following IHC staining. Therefore, DNA methylation analysis was performed on 844 samples, including 113 controls, 565 vHSIL, 80 vLSIL, 43 dVIN, 31 non-dysplastic lesions, 3 VSCC and 9 inconclusive cases. Depending on the multiplex assay, between 810 and 817 samples (96-97%) had a valid methylation result (Figure 1).

All 12 markers showed a significant difference in DNA methylation levels across the various disease categories (p<0.001, Kruskal-Wallis test). Overall, methylation levels increased with increasing severity of disease (Figure 1). Significantly higher methylation levels were found in high-grade VIN cases (vHSIL and dVIN) compared to healthy controls. For all markers, except *CADM1* and *PHACTR3*, significantly higher methylation levels were also found in high-grade VIN cases compared to non-dysplastic cases and vLSIL. Interestingly, seven out of 12 markers (i.e. *ZNF582, GHSR, CADM1, MAL, FAM19A4, PHACTR3* and *PRDM14*) showed significantly higher methylation levels in dVIN compared to vHSIL. A high variety in methylation levels was found in vHSIL, whereas methylation levels in dVIN were more consistently high. Although there was a slight increase in methylation levels from controls towards the non-dysplastic and the vLSIL groups, these differences were mostly not statistically significant.

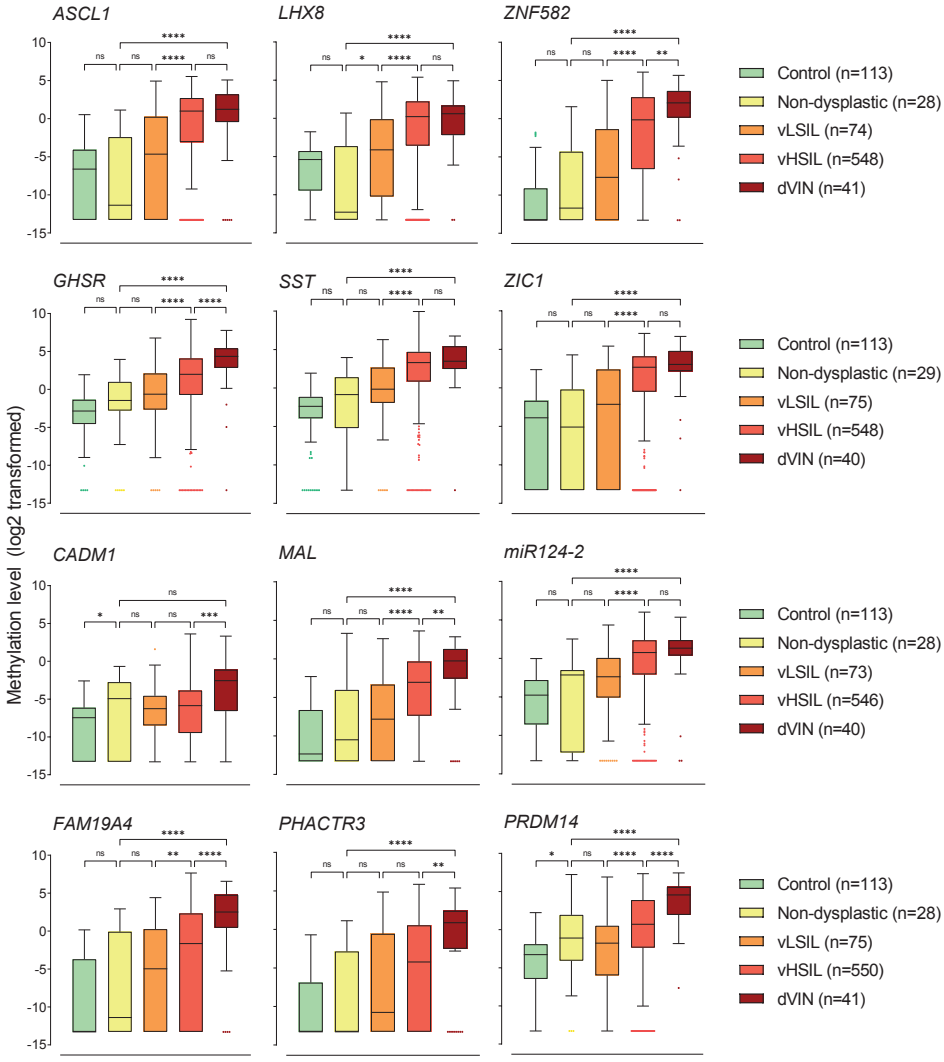

Figure 1. DNA methylation levels of 12 genes across five vulvar disease categories.

Number of valid cases per disease category are presented for each multiplex in the legend. Differences between categories were tested by the Kruskal-Wallis test, followed by pairwise post-hoc Mann-Whitney *U* testing with Bonferroni correction for multiple comparisons; ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Performance individual markers

All markers showed a moderate to good individual performance to distinguish high-grade VIN from healthy controls. AUC values ranged from 0.67 to 0.90, with nine markers showing an AUC above 0.80 (Table 2). The best performing marker was *SST*

Table 2. Diagnostic performance for high-grade VIN detection of the 12 individual DNA methylation markers

Methylation marker	AUC (95% CI)	Sensitivity (%)	Specificity (%)
SST	0.90 (0.87 – 0.92)	80	98
miR124-2	0.87 (0.85 – 0.90)	69	98
GHSR	0.87 (0.84 – 0.89)	68	97
ZNF582	0.85 (0.82 – 0.88)	73	94
ZIC1	0.84 (0.81 – 0.87)	68	96
ASCL1	0.84 (0.81 – 0.87)	72	97
LHX8	0.83 (0.80 – 0.86)	72	96
MAL	0.81 (0.78 – 0.84)	60	98
PRDM14	0.80 (0.77 – 0.84)	65	91
FAM19A4	0.76 (0.72 – 0.79)	60	89
PHACTR3	0.72 (0.67 – 0.76)	61	81
CADM1	0.67 (0.63 – 0.72)	44	92

Results are ordered based on highest AUC value. Sensitivity and specificity based on Youden's *J*-index.
Abbreviations: AUC, area under the curve; VIN, vulvar intraepithelial neoplasia.

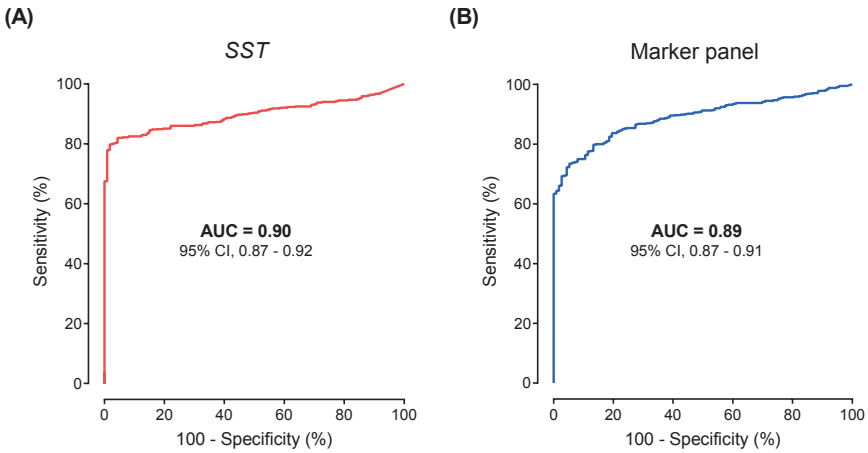

Figure 2. Performance of single marker *SST* and the marker panel for detection of high-grade VIN.
A. Receiver operating curve (ROC) with corresponding area under the curve (AUC) value for *SST*;
B. ROC and AUC for the marker panel, including genes *ZNF582, SST, miR124-2*.

(AUC 0.90, 95% CI; 0.87 – 0.92) with a sensitivity of 80% and specificity of 98% for high-grade VIN detection at Youden's index (*J*-threshold ≥0.78) (Figure 2A). Using this threshold, *SST* classified 100% (2/2) of VSCC, 95% (37/39) of dVIN, 79% (425/541) of vHSIL, 47% (34/72) of vLSIL, 38% (11/29) of non-dysplastic and 2% (2/113) of controls as methylation positive (Figure 3A). Upon internal five-fold cross-validation a nearly identical AUC of 0.89 (0.87 – 0.92, 95% CI) was obtained, supporting its robustness.

Marker panel selection for optimal high-grade VIN detection

Multivariate logistic regression with backward selection for high-grade VIN detection yielded an optimal marker panel consisting of *ZNF582*, *miR124-2* and *SST*, with an AUC value of 0.89 (0.87 – 0.91, 95% CI) (Figure 2B). The Youden's index provided a sensitivity of 73% and specificity of 95% (*J*-threshold ≥0.68). Using this threshold, the marker panel classified 50% (1/2) of VSCC, 87% (34/39) of dVIN, 72% (392/541) of vHSIL, 36% (26/74) of vLSIL, 21% (6/28) of non-dysplastic and 5% (6/113) of controls as methylation positive (Figure 3B). Internal five-fold cross-validation gave a comparably high cross-validated AUC value of 0.87 (0.84 – 0.89, 95% CI).

Discussion

In this study we validated the high accuracy of DNA methylation markers for the detection of high-grade VIN in a large historical cohort. Highest accuracy for detection of high-grade VIN was achieved using solely *SST* as a single marker (AUC 0.90). Selection of a marker panel, including *ZNF582*, *SST* and *miR124-2*, resulted in a comparably high accuracy for the detection of high-grade VIN (AUC 0.89). In accordance with our earlier findings, we found a strong correlation between methylation levels and severity of vulvar diagnosis in both HPV-associated and HPV-independent lesions.[16] Particularly for challenging HPV-independent precursor lesions that mimic reactive or inflammatory non-dysplastic lesions, the use of these methylation assays can be very useful as an adjunct diagnostic tool.

Most markers showed significantly higher methylation levels in dVIN compared to vHSIL, which is consistent with the higher cancer risk in patients with dVIN.[6, 7] Interestingly, a more heterogeneous methylation pattern was observed within the vHSIL group, likely reflecting a varying cancer risk. A previous study by our research group also identified *SST* as one of the best performing methylation markers for cancer risk stratification in HPV-induced anal lesions.[23] Longitudinal studies are needed to further explore the prognostic value of methylation markers for cancer risk stratification in vulvar lesions as well.

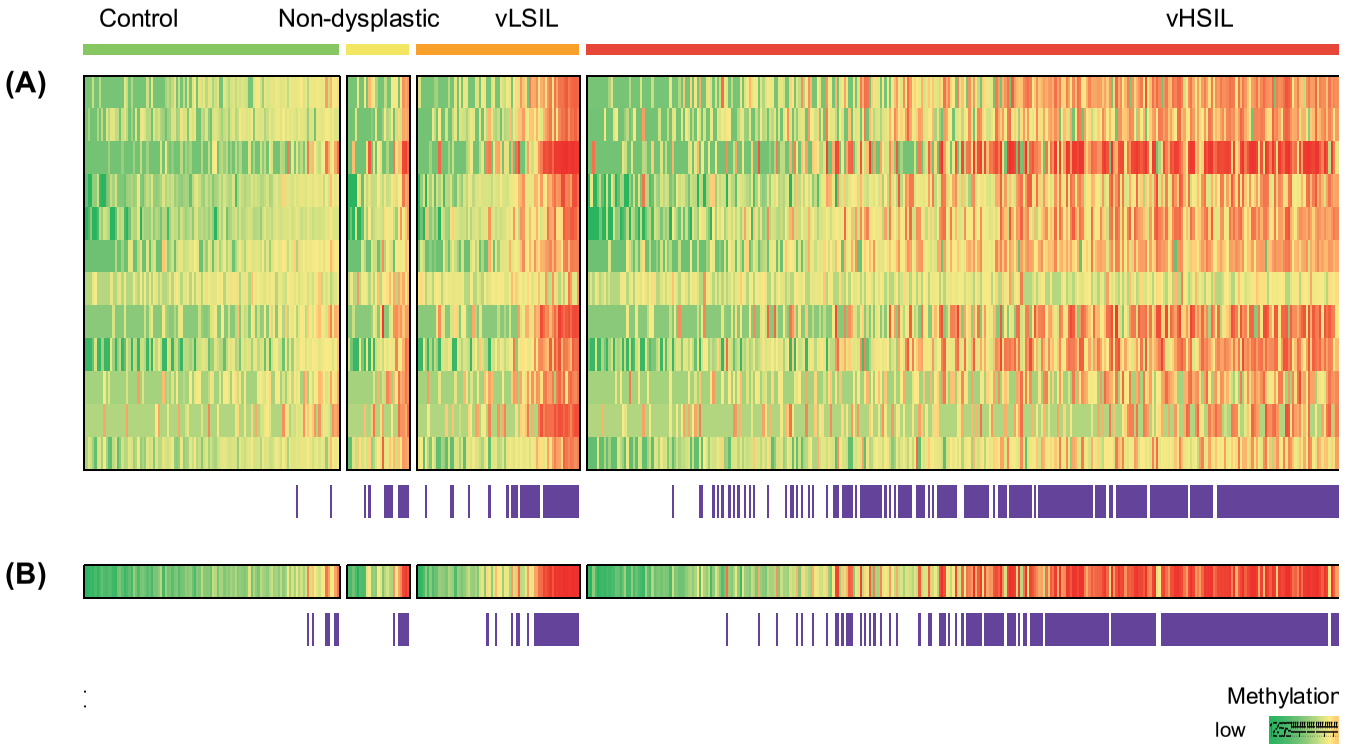

Figure 3. DNA methylation patterns across all tested samples. (A) Methylation patterns of all individual markers tested (*ASCL1*, *LHX8*, *ZNF582*, *GHSR*, *SST*, *ZIC1*, *CADM1*, *MAL*, *miR124-2*, *FAM19A4*, *PHACTR3* and *PRDM14*) for all samples across five vulvar disease categories (control, non-dysplastic, vLSIL, vHSIL and dVIN). Per sample, the methylation result is presented in colour according to the predicted probability result from univariate logistic regression analysis for high-grade VIN detection (low levels in green, high levels in red). Purple boxes represent cases classified as methylation positive by single marker *SST* using *J*-threshold ≥0.78; (B) DNA methylation pattern of the marker panel (*ZNF582*, *SST, miR124-2*) selected by multivariate logistic regression analysis with backward selection. Samples detected as methylation positive by the marker panel are displayed in purple, using *J*-threshold ≥0.68.

Even though we tested 12 DNA methylation markers primarily known to be associated with HPV-induced carcinogenesis,[14, 15] we acknowledge that the most common etiopathogenic pathway of development towards VSCC is independent of HPV. While to a certain extent different epigenetic alterations may be expected in HPV-associated versus HPV-independent vulvar premalignant lesions, a recent genome wide methylation study on vulvar cancers also shows overlapping DNA methylation events between these two pathogenic pathways.[24] In this study, Dasgupta et al. identified 199 genes which were differentially methylated in VSCC compared to healthy vulvar tissue. These included five out of the 12 markers that were analysed in the present study, including markers *SST* and *ZNF582* from our marker panel. Since the study by Dasgupta et al. included more HPV-independent VSCC than HPV-associated VSCC (15 versus 3 cases, respectively), this further supports the potential of these markers for the detection of HPV-independent vulvar lesions.[24] In addition, some of our markers tested are known as pan-cancer markers, including *GHSR* and *SST*.[25, 26] These markers are known to be hypermethylated in a variety of cancers, and therefore a potential biomarker for lesions arising from different pathogenic pathways.[26, 27]

Consistently, *SST* was also the best performing individual marker in our previous study to distinguish high-grade VIN without adjacent VSCC from healthy vulvar controls (AUC 0.93), irrespective of HPV status.[16] *SST* encodes a growth-regulatory peptide hormone (somatostatin) which is involved in the regulation of cell migration.[28] It acts as a tumour suppressor gene in multiple cancer types, including those of the gastrointestinal tract and prostate, as well as in melanoma. Somatostatin binds to specific somatostatin binding receptors (SSTRs) which are expressed in various tumour tissues throughout the human body.[29, 30] Binding of somatostatin to SSTR-expressing tumour cells can lead to tumour regression by inducing apoptosis.[31] *ZNF582* encodes a zinc finger protein which is presumed to be involved in transcriptional regulation, but literature on its specific function is lacking.[32] MicroRNA 124-2 has a tumour suppressive function in HPV-positive cells and has been demonstrated to inhibit proliferation and migration of cervical cancer cells.[33] While for *miR124-2* silencing of its expression by DNA methylation has been confirmed, further data on *SST* and *ZNF582* expression regulation as well as a potential tumour suppressive function are still to be awaited.

To our knowledge, this is the largest series of vulvar lesions in which DNA methylation patterns have been studied. Given the fact that high-grade VIN is a relative rare disease, the extensive analyses of such a large series is unique. Cases were selected from a population-based historical cohort, reducing the risk of selection bias. Histopathologic review of cases was performed by two experts in vulvar pathology,

and categorization of vulvar lesions included immunohistochemical markers $p16^{INK4a}$, p53 and MIB-1 and HPV testing, resulting in a well-defined series of HPV-associated and HPV-independent vulvar lesions.

Even so, our study has some limitations. Firstly, histological reassessment was based on newly cut H&E slides. However, some tissue blocks had limited residual tissue of the original biopsy or excision. Therefore, there is a possibility that the high-grade dysplastic tissue, on which the original diagnosis was based, was no longer present in the tissue block. With this in mind, the methylation positive cases in lesions downgraded to non-dysplastic or vLSIL after revision could partly be explained by the concept of field cancerization, also known as the field effect. This involves the presence of tumour-specific epigenetic changes in the cells surrounding and adjacent to tumour cells.[34] Additional studies on DNA methylation markers in healthy and benign vulvar lesions adjacent to VSCC are needed to confirm this.

Moreover, emphasis should be given to the healthy control group when appreciating the high specificity results. Healthy vulvar tissues were collected from patients who had undergone aesthetic or reconstructive genital procedures. Thus, strictly speaking, the study cohort does not fully represent patients with vulvar symptoms who visit the gynaecologist in general practice. As a result, there is a large age difference between the control cohort and the other disease categories. Studies have shown that certain regions of the genome, such as the promotor regions of genes involved in the regulation of cell growth and division, tend to become increasingly methylated with age. However, the influence of age on methylation is presumably much weaker than the strong biological processes involved in vulvar carcinogenesis.[35]

In conclusion, DNA methylation markers demonstrate a high accuracy for detection of high-grade VIN and can be used as an adjunct diagnostic tool for challenging lesions. These biomarkers, in combination with other clinical parameters, have the potential to optimize the identification of vulvar lesions with a high cancer risk, allowing personalized management for affected patients. Longitudinal studies including clinical follow-up data are ongoing to investigate the prognostic value of these markers in high-grade precursor lesions and its mimickers.

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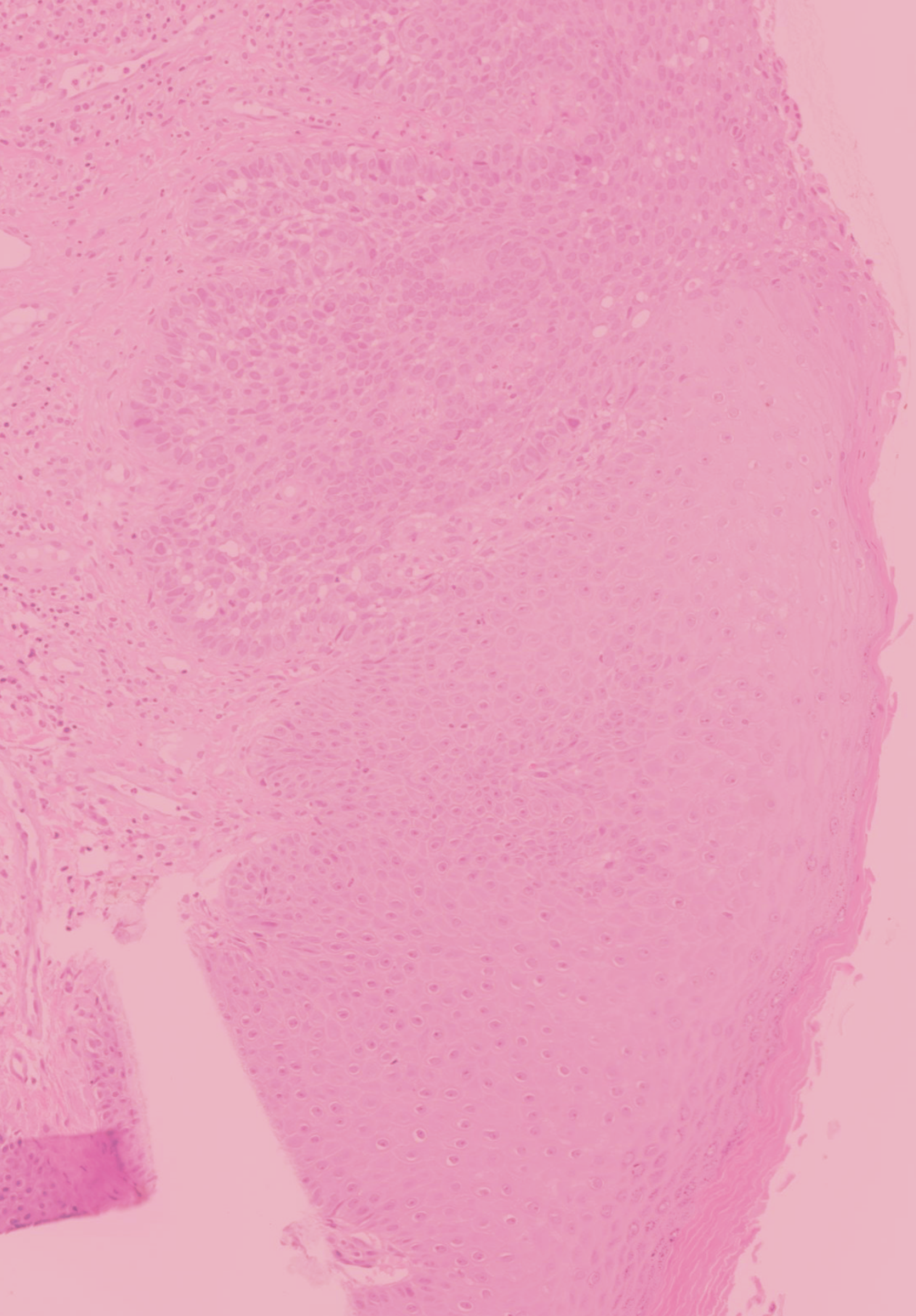
Supplementary Table 1. Immunohistochemistry and HPV genotyping results for HPV-associated and HPV-independent vulvar disease categories

		HPV-ASSOCIATED						HPV-INDEPENDENT					
		vLSIL		vHSIL		VSCC		Non-dysplastic		dVIN		VSCC	
		(n=87)		(n=575)		(n=3)		(n=31)		(n=44)		(n=1)	
		n	%	n	%	n	%	n	%	n	%	n	%
P16^{INK4a}	Negative	59	67.8	6	1.0	0	0.0	31	100.0	43	97.7	1	100.0
	Block positive	28	32.2	568	98.8	3	100.0	0	0.0	1	2.3	0	0.0
P53	Wild-type	87	100.0	571	99.8	3	100.0	31	100.0	10	22.7	1	100.0
	Mutant	0	0.0	1	0.2	0	0.0	0	0.0	34	77.3	0	0.0
MIB-1	Normal	20	23.0	2	0.3	0	0.0	19	61.3	6	13.6	0	0.0
	Increased	67	77.0	573	99.7	3	100.0	12	38.7	38	86.4	1	100.0
Tested for high-risk HPV		80	92.0	565	98.3	2	66.7	31	100.0	43	97.7	1	100.0
	Valid test result	64	80.0	556	98.4	1	50.0	10	32.3	32	74.4	1	100.0
	high-risk HPV positive	45	70.0	550	98.9	1	100.0	0	0.0	4	12.5	0	0.0
Tested for low-risk HPV		16	18.4	8	1.4	0	0.0	1	3.2	15	34.1	0	0.0
	Valid test result	16	100.0	8	100.0	-	-	1	100.0	15	100.0	-	-
	low-risk HPV positive	13	81.3	4	50.0	-	-	0	0.0	1	6.7	-	-

Abbreviations: dVIN, differentiated vulvar intraepithelial neoplasia; HPV, human papillomavirus; vHSIL, vulvar high-grade squamous intraepithelial lesion; vLSIL, vulvar low-grade squamous intraepithelial lesion; VSCC, vulvar squamous cell carcinoma.



CHAPTER 8

DNA methylation testing for vulvar cancer risk stratification in patients with high-grade vulvar intraepithelial neoplasia: a population-based cohort study

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Abstract

High-grade vulvar intraepithelial neoplasia (VIN) is the precursor of vulvar cancer and is divided into human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesion (HSIL) and HPV-independent VIN, often clinically referred to as differentiated VIN (dVIN) and associated with vulvar dermatoses, usually lichen sclerosus. Surgical treatment of high-grade VIN often leads to genital deformity, reduced quality of life and reduced sexual function, which has a major impact on quality of life. To optimize clinical management, accurate biomarkers providing information on the cancer risk of high-grade VIN are needed.

To investigate the prognostic value of a three-gene methylation marker panel and other potential risk factors for the risk of progression to cancer in patients with HSIL and dVIN.

From a population-based cohort of patients diagnosed with high-grade VIN, patients with a histopathological confirmed diagnosis of HSIL (n=578) and dVIN (n=46) were selected. All lesions were tested for a three-gene methylation panel including genes *ZNF582, SST,* and *miR124-2*. The vulvar cancer risk and the prognostic value of methylation, age, HPV genotype, p53 immunohistochemistry status, and presence of lichen sclerosus were estimated by Kaplan-Meier and Cox regression, respectively.

In patients with HSIL, a positive methylation status was identified as the only prognostic factor for vulvar cancer development (HR 4.87; 95%CI 1.20 – 21.45). The prognostic value of methylation remained present when selecting patients who did not receive radical surgical excision as their primary treatment. In this group, the 5-year cancer risk was 7.7% in methylation-positive HSIL and 1.4% in methylation-negative HSIL (p=0.008). In patients with dVIN, p53 status was the sole prognostic risk factor for progression to cancer (HR 7.67; 95% CI 1.78 – 33.08).

In patients with vulvar HSIL, the three-gene methylation test is a valuable prognostic tool for cancer risk stratification. Patients with methylation-negative HSIL carry a low cancer risk and can be safely managed without radical treatment, reducing morbidity and improve quality of life.

Introduction

Precursor lesions of vulvar cancer can be categorized into human papillomavirus (HPV)-associated vulvar high-grade squamous intraepithelial lesion (HSIL) and HPV-independent vulvar intraepithelial neoplasia (VIN), the latter commonly referred to as differentiated VIN (dVIN).

HSIL is the most common vulvar precursor lesion with a peak incidence in patients between 40 and 50 years of age. HSIL patients often present with multifocal and multicentric disease, i.e. lesions at other anogenital sites, such as the cervix, anus or vagina.[1] Treatment options are excision, laser vaporization or topical application with imiquimod.[2] The 5-year vulvar cancer risk after treatment is 4.5%, whereas patients with untreated HSIL have reported cancer risks of 9 and 88%.[3-5] Surgical treatment often leads to disfigurement, reduced quality of life and impaired sexual function.[6] The treatment of vulvar HSIL is challenging as the aim is to prevent recurrences and progression to vulvar cancer while minimizing treatment-related harms. In order to guide clinicians in their management strategy, accurate cancer risk assessment in patients with HSIL is crucial. However, to date, objective prognostic biomarkers are lacking.[7-9]

DVIN is diagnosed much less frequently than HSIL, presents mainly in older patients and is associated with vulvar dermatoses, most commonly lichen sclerosus (LS).[10,11] The cancer risk of dVIN is very high compared to HSIL. In fact, patients with p53 mutant dVIN have the highest 5-year cancer risk of 63.3%, necessitating aggressive excisional treatment and close monitoring.[5] Patients with p53 wild-type dVIN have a moderate 5-year cancer risk of 12.5%, and further stratification of these patients based on cancer risk seems warranted.[5]

Methylation of tumour suppressor genes has proven to be valuable in distinguishing high-grade VIN from non-dysplastic vulvar lesions.[12] In a previous study of 864 vulvar tissues, a methylation marker panel consisting of genes *ZNF582, SST and miR124-2,* was identified, demonstrating a high accuracy for the detection of both HSIL and dVIN.[13]

The aim of the current study was to investigate the prognostic value of the three-gene methylation marker panel and other risk factors for progression to cancer, such as age, presence of lichen sclerosus, p53 immunohistochemistry status, and HPV genotype, in patients with HSIL and dVIN.

Patients and methods

Study population and data collection

This study is based on a large, longitudinal population-based cohort series of 751 patients with a primary diagnosis of high-grade VIN between 1991 and 2011, without prior or concurrent vulvar cancer. All baseline high-grade VIN of this cohort were characterized by $p16^{INK4a}$, p53 and ki-67 immunohistochemistry (IHC) and HPV DNA testing, as previously described.[5,10] For the current study, only cases with a histopathological revised diagnosis of high-grade VIN were selected from this series and further categorized as HSIL (n=578) and dVIN (n=46; including 30 p53 mutant and 16 p53 wild-type cases), resulting in a total study cohort of 624 patients.[5]

Pathology reports up to 2020 were available to extract age at first high-grade VIN diagnosis and date of progression to vulvar cancer. High-grade VIN categorization was based on histopathological reassessment, integrating the results of immunohistochemistry ($p16^{INK4A}$, p53 and ki-67) and HPV testing, as described before.[5] DNA methylation analysis was performed as described previously.[5,13] In short, DNA was isolated from the formalin-fixed paraffin embedded (FFPE) tissue blocks and subsequently modified by bisulphite conversion. Modified DNA was analysed for markers *ZNF582*, *SST* and *miR124-2*, using multiplex quantitative methylation-specific polymerase chain reactions (qMSP).[13] Cases were classified as methylation-positive or -negative based on a previously determined threshold for the three-gene methylation marker panel.[13] P53 staining was categorized as wild-type (scattered or mid-epithelial staining with basal sparing) or mutant (aberrant positive nuclear or cytoplasmic staining, or complete absent staining/null-pattern).[5,14,15]

In addition, a prior or concurrent histopathologic diagnosis of LS was extracted from the pathology reports. Information on primary treatment was collected from the pathology reports and available clinical medical records. Primary treatment was categorized into 'radical excision' or 'non-radical treatment'. 'Radical excision' was defined as excisional surgery with histopathologically negative resection margins without residual or multifocal high-grade VIN within 6 months after the initial biopsy. The remaining treatment modalities were classified as 'non-radical treatment', including excisional surgery with histopathologically positive margins, laser vaporization, imiquimod, other non-surgical treatment modalities and no treatment within 6 months after biopsy. If radicality could not be determined with certainty, it was recorded as missing. For dVIN patients, no separate analyses were done for treatment modality.

Statistical analysis

The primary study endpoint was progression to vulvar cancer during follow-up. Time to vulvar cancer was defined as the time between baseline high-grade VIN and vulvar cancer diagnosis. For patients without cancer, time was censored at the closing date of the database (28 October 2020) or the expected date of death, whichever occurred first. The expected date of death was calculated using age-and calendar year-dependent life expectancy tables from Statistics Netherlands (StatLine, CBS).[16]

Cumulative cancer risks and 95% confidence intervals (CI), stratified by methylation status, were estimated by the Kaplan-Meier method. The association between cancer risk and methylation status was evaluated by the Breslow (Generalized Wilcoxon) test. Hazard ratios (HR) for the associations between risk factors and progression to cancer within five years were estimated using univariable and multivariable Cox regression. For patients with HSIL, risk factors included methylation status, age (continuous) and HPV genotype (HPV16 positive or not). For patients with dVIN, risk factors included p53 IHC status, methylation status, age (continuous) and presence of LS. Age and statistically significant risk factors in the univariable analysis were included in the multivariable analysis.

Missing data due to insufficient tissue for HPV DNA and methylation testing were handled by multiple imputation with fully conditional specification (MCMC) and predictive mean matching (PMM). Twenty-four HSIL patients had a missing methylation result, 17 HSIL patients had a missing HPV16 result and 17 HSIL patients had a missing result of both HPV16 and methylation status. DVIN patients had no missing results.

The associations between methylation and type of treatment were determined using Pearson Chi-Square tests. The level of statistical significance was set at 0.05. Statistical analyses were performed using IBM SPSS Statistics software for Windows version 28.0 (IBM Corporation, Armonk, NY) and graphs were produced in GraphPad Prism 9.

Results

Patient characteristics

Characteristics of the study cohort of 624 patients with high-grade VIN are shown in **Table 1** and included 578 (92.6%) HSIL patients and 46 (7.4%) dVIN patients. Patients with dVIN had a higher median age at diagnosis and more often LS than patients with HSIL (*p*-values <0.001). The methylation test was positive in 70.9% of HSIL and 84.8%

of dVIN (p=0.044). Primary treatment did not involve radical excisional treatment for at least 94.6% of patients of whom radicality could be assessed, and there was no significant difference between HSIL and dVIN patients (p=0.443). Of the 454 HSIL patients who received non-radical treatment, 234 patients (51.5%) underwent a non-radical excision, 15 patients (3.3%) received laser therapy, 13 patients (2.9%) received imiquimod and 54 patients (11.9%) were not treated within 6 months after the initial biopsy. Of the 28 dVIN patients who received non-radical treatment, 14 patients (50%) underwent a non-radical excision and 9 patients (32.1%) were not treated within 6 months after the initial biopsy. Remaining patients either received another non-surgical treatment modality or there was incomplete clinical data available. A full overview of the various treatment modalities is provided in **Supplementary Figure S1**. Median follow-up time of patients without vulvar cancer was significantly longer for HSIL patients compared to dVIN patients: 16.3 versus 11.0 years (p<0.001).

Table 1. Characteristics of the study cohort

	HSIL	**DVIN**
	n (%)	**n (%)**
	578 (92.6)	**46 (7.4)**
Patient characteristics		
Age (years)		
Median	45	72
Mean (± SD)	46.6 (14.3)	70.3 (12.7)
Range	17-90	35-92
Lichen sclerosus		
No	567 (98.1)	23 (50.0)
Yes	11 (1.9)	23 (50.0)
Primary treatment modality		
Non-radical treatment	454 (78.5)	38 (82.6)
Radical excision	27 (4.7)	1 (2.2)
Missing	97 (16.8)	7 (15.2)
Lesion characteristics		
Methylation status		
Negative	168 (29.1)	7 (15.2)
Positive	410 (70.9)	39 (84.8)
HPV16		
Negative	106 (18.3)	39 (84.8)
Positive	472 (81.7)	7 (15.2)
Immunohistochemistry p53		
Wild-type	577 (99.8)	16 (34.8)
Mutant	1 (0.2)	30 (65.2)

DVIN, differentiated vulvar intra-epithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion.

Cancer risk assessment in patients with vulvar HSIL

Progression to vulvar cancer occurred in 26/578 (4.5%) of HSIL patients within five years and in 61/578 (10.6%) of patients within the total follow-up period of 29.6 years. Methylation status was only weakly associated with vulvar cancer risk over the total follow-up period (Breslow p=0.050), yet strongly associated with cancer risk after five years (Breslow p=0.013) (**Figure 1**). Besides, the median time to progression to vulvar cancer differed significantly between methylation-positive and –negative HSIL (i.e., 4.9 and 12.6 years, respectively). Notably, none of the 168 patients with methylation-negative HSIL developed vulvar cancer in the first four years of follow-up.

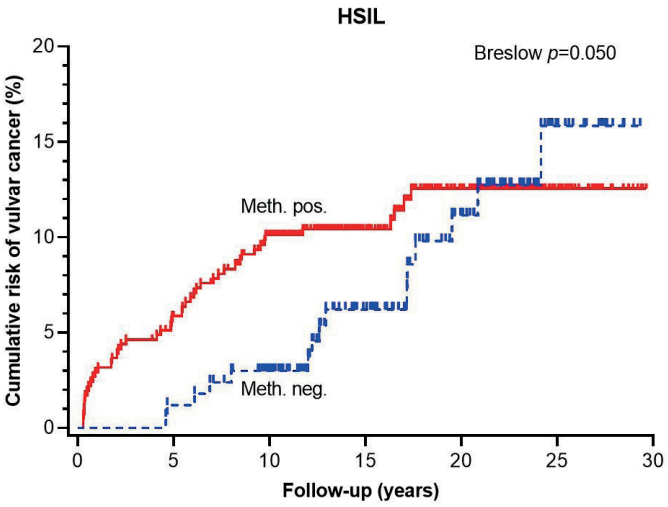
Univariable and multivariable Cox regression analyses for progression to cancer within five years are shown in **Table 2**. Only methylation was significantly associated with cancer risk, with a HR of 4.87 (95% CI, 1.20 – 21.45) in univariate analysis and an age-adjusted HR of 4.50 (95% CI, 1.04 – 19.43). Similar HRs were obtained when cases with missing HPV DNA and methylation data were not imputed, with a p-value of 0.033 for the effect of methylation.

Five-year cancer risk, stratified for type of primary treatment and methylation status, is shown in **Table 3**. A five-year cancer risk of 5.7% (95% CI, 3.4 – 7.8) was observed in patients who did not receive primary radical surgical excision, representing 94.4% of the patients in whom radicality of primary treatment could be assessed. In this group the cancer risk was significantly higher in patients with methylation-positive HSIL compared to patients with methylation-negative HSIL (7.7% versus 1.4%, respectively, p=0.008).

Table 2. Prognostic factors for progression to vulvar cancer within five years in patients with HSIL

			Univariable analysis			Multivariable analysis		
		n	HR	(95% CI)	p-value	HR	(95% CI)	p-value
Methylation	**Negative**	168						
	Positive	410	4.87	(1.20-21.45)	**0.027**	4.50	(1.04-19.43)	**0.044**
HPV16	**Negative**	106						
	Positive	472	1.74	(0.52-5.79)	0.368			
Age, per year		578	1.02	(0.996-1.05)	0.106	1.01	(0.99-1.04)	0.347

Abbreviations: CI, confidence interval; HPV, human papillomavirus; HR, hazard ratio; HSIL, high-grade squamous intraepithelial lesion.



Numbers at risk							
Meth. pos.	410	381	344	202	111	39	0
Meth. neg.	168	165	150	98	61	17	0
Cumulative risk of vulvar cancer (%)							
Meth. pos.		6.1	10.1	10.4	12.5	12.5	
Meth. neg.		1.8	3.0	6.2	11.1	15.8	

Figure 1. Cumulative risk of vulvar cancer in patients with HSIL stratified for methylation status.
Abbreviations: HSIL, high-grade squamous intraepithelial lesion; Meth., methylation.

Cancer risk assessment in patients with dVIN

Progression to vulvar cancer occurred in 21/46 (45.7%) of dVIN patients within five years and in 25/46 (54.3%) of patients within the total follow-up period of 23.3 years. Methylation status was not related to vulvar cancer risk (Breslow p=0.322, **Figure 2**), and the median time to cancer did not differ between methylation-negative and -positive dVIN (i.e., 2.7 and 1.9 years, respectively, p=0.446). Of all risk factors, including methylation status, p53 IHC status, presence of LS, and age, only p53 status was independently associated with development to cancer within five years after diagnosis of dVIN (HR 7.45, 95% CI, 1.72 – 32.22, p=0.007; **Table 4**).

Methylation positivity was observed in 27/30 (90.0%) of p53 mutant dVIN and in 12/16 (75.0%) of p53 wild-type dVIN (p=0.177). Within the p53 wild-type dVIN, none of the methylation-negative cases progressed to cancer (0/4) within 10 years, whereas 41.7% (5/12) of the methylation-positive cases did show progression to cancer within five years. A statistically significant difference could not be demonstrated.

In patients with methylation-negative dVIN (n=7), a potential association was detected between p53 IHC and progression to cancer, as progression to cancer was observed in 2/3 p53 mutant cases and in 0/4 p53 wild-type cases within 10 years (*p*=0.053).

Table 3. Five-year vulvar cancer risk, stratified for methylation status of baseline HSIL and type of primary treatment

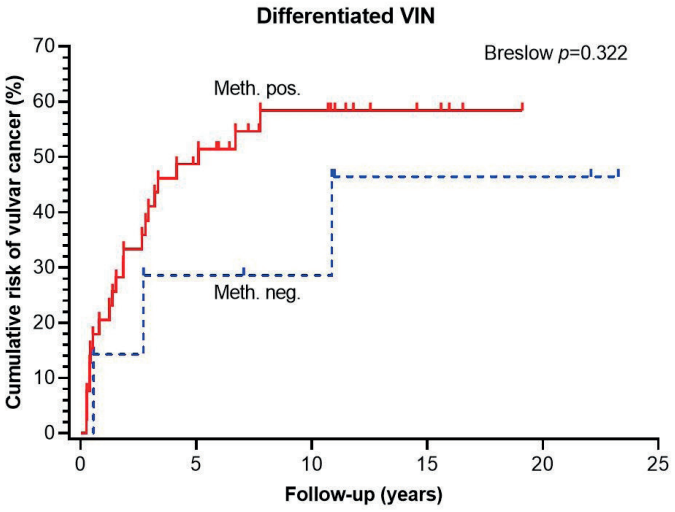
			Progression to cancer within five years		
	Methylation	**Total**	**n**	**(%)**	**Breslow *p*-value**
Total patient group	All	481	26	(5.4)	
	Positive	337	24	(7.4)	0.010
	Negative	144	2	(1.4)	
Non-radical treatment	All	454	26	(5.7)	
	Positive	314	24	(7.7)	0.008
	Negative	140	2	(1.4)	
Radical excision	All	27	0	(0.0)	
	Positive	23	0	(0.0)	NA
	Negative	4	0	(0.0)	

Abbreviations: HSIL, high-grade squamous intraepithelial lesion. Only cases with known type of primary treatment were included in this table.

Table 4. Prognostic factors for risk of progression to vulvar cancer within five years in patients with dVIN

		Univariable analysis				Multivariable analysis		
		n	**HR**	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**
Methylation	**Negative**	7						
	Positive	39	1.97	(0.46-8.48)	0.361			
P53 IHC status	**Wild-type**	16						
	Mutant	30	7.67	(1.78-33.08)	**0.006**	7.45	(1.72-32.22)	**0.007**
Lichen sclerosus	**Absent**	23						
	Present	23	0.82	(0.35-1.93)	0.649			
Age, per year		46	0.98	(0.95-1.02)	0.298	0.99	(0.95-1.02)	0.473

Abbreviations: CI, confidence interval; dVIN, differentiated vulvar intraepithelial neoplasia; HPV, human papillomavirus; HR, hazard ratio; IHC, immunohistochemistry.



Numbers at risk						
Meth. pos.	39	18	10	3	0	0
Meth. neg.	7	4	4	2	2	0
Cumulative risk of vulvar cancer (%)						
Meth. pos.		53.2	58.4	58.4		
Meth. neg.		28.6	46.4	46.4	46.4	

Figure 2. Cumulative risk of vulvar cancer in patients with dVIN stratified for methylation status.
Abbreviations: dVIN, differentiated vulvar intraepithelial neoplasia; Meth., methylation.

Discussion

In this population-based cohort including 624 patients with high-grade VIN, the prognostic value of DNA methylation for cancer risk stratification was assessed. Methylation was the only prognostic factor in patients with HSIL, with a 4.9-fold higher 5-year cancer risk in methylation-positive versus methylation-negative HSIL patients. In the subgroup that received non-radical primary treatment, which was the majority of the HSIL cohort (94.4%), these promising results were maintained. None of 168 patients with methylation-negative HSIL progressed to cancer in the first four years of follow-up, which supports conservative management to reduce morbidity with maintenance of sexual function and quality of life. In patients with dVIN, p53 status was identified as the only prognostic risk factor for progression to cancer with a 7.7 fold higher 5-year cancer risk in p53 mutant versus p53 wild-type dVIN patients.

HSIL

This study showed that testing for the three-gene methylation marker panel has substantial value for determining cancer risk in HSIL patients, with methylation-positive HSIL having nearly a five-fold higher 5-year cancer risk compared to methylation-negative HSIL. In our series, methylation remained discriminative for cancer risk in HSIL patients up to 10 years. This suggests that although hypermethylation of tumour suppressor genes is an early event in (ano)genital precursor lesions, it is not prognostic for cancer developing 20 years later, as demonstrated in the Kaplan Meier analysis in this study.[13,17,18] Currently there is only one study that has examined DNA methylation in relation to progression to invasive cancer in (ano)genital precursors. In this longitudinal study with 40 biopsies of 10 patients with anal HSIL preceding cancer, all HSIL displayed high methylation levels similar to the anal cancers up to 2.5 years before cancer diagnosis.[19] Taken together with our findings, these results underscore the prognostic value of methylation for cancer risk stratification of (ano)genital precursor lesions.

In our series, HPV16 was not identified as a prognostic factor associated with vulvar cancer in HSIL. This is most likely due to the fact that almost 90% of HSIL were HPV16 positive. Age was neither identified as a significant prognostic factor.

Nearly 30% of HSIL patients were methylation-negative and only 2/168 (1.2%) of these patients progressed to cancer within five years, respectively after 4.6 and 4.7 years. Both cancers were small in size, with only a minimal micro-invasive focus. Both patients had HPV16 and $p16^{INK4a}$-positive multifocal HSIL, and received multiple, non-radical treatments before cancer diagnosis. At time of cancer diagnosis, both patients had multicentric disease. Taken together, these data indicate that patients with methylation-negative HSIL can be reassured of their low risk of progression to cancer. Subsequently, this information may also be of added value in the choice of treatment for HSIL (i.e., imiquimod, laser therapy, surgical excision). Considerations for treatment choice include localization and size of the lesion, as well as patient characteristics and preferences. Methylation could act as an additional tool to aid in shared-decision making of HSIL patients. Our data imply that methylation-negative patients could safely be treated conservatively, i.e. 'non-radically', through treatment modalities such as imiquimod or laser vaporization. This is supported by the results of a recent randomized, non-inferiority trial, demonstrating imiquimod is a safe and effective alternative to excisional surgery.[20] Such conservative treatment will help preserve (ano)genital structural anatomy, thereby diminishing psychological distress and improving quality of life. In addition, methylation could assist in deciding how to treat patients with multicentric disease, which is estimated to affect 25 – 66%

of patients with VIN.[21,22] A positive methylation result may require more urgent treatment of concomitant (ano)genital precursors, while a methylation-negative result may allow for watchful waiting. Of note, a wait-and-see policy in patients with methylation-negative HSIL is possibly safe, but cannot be concluded from our data, as most patients of the study cohort received treatment. Patients with methylation-positive HSIL had a 5-year cancer risk of 5.9% in this study, which does not justify a change in the current standard of care. This includes sufficient biopsies to rule out invasive disease, no re-excision after positive histopathological surgical margins, and life-long surveillance, the latter being supported by a persistent increased cancer risk up to 30 years as observed in this cohort.[5,10]

DVIN

In the last decade, p53 and p16^{INK4a} immunostaining have been increasingly used as diagnostic adjuncts in the classification of vulvar cancer and its precursors.[5,23] We recently showed that p53 is particularly important in HPV-independent precursors, as p53 mutant dVIN has a substantial higher 5-year cancer risk than p53 wild-type dVIN, respectively 63.3% versus 12.5%.[5]

In this study, the only independent factor associated with progression to vulvar cancer in patients with dVIN, was mutant p53 status. Although the added value of p53 as prognostic factor in dVIN is becoming increasingly acknowledged, it is not yet a formal recommendation and evidence is limited.[5,24,25] As solitary dVIN is a rare disease, longitudinal studies on cancer development are scarce.[26] In vulvar cancer patients, increased recurrence rates have been observed in patients with p53 mutant dVIN in the surgical margins of the resection specimens.[27,28] Also, higher recurrence rates and worse overall- and disease free survival have been shown for patients with p53 mutant HPV-independent vulvar cancer compared to patients with p53 wild-type HPV-independent cancer.[27-34] These results are in line with the findings in this study, in which p53 mutant dVIN displayed a 7.7-fold higher cancer risk, indicating the need for aggressive treatment and close follow-up of these patients. The lower cancer risk in p53 wild-type dVIN could imply added value of methylation in cancer risk stratification of these patients. Although the limited size of patients with p53 wild-type dVIN in this study (n=16) made it difficult to draw firm conclusions, the results showed a nearly significant association between methylation status and cancer risk. These results are potentially important given that there are no adjunct tools to distinguish progressive p53 wild-type dVIN from non-progressive p53 wild-type dVIN. Moreover, methylation could serve as a diagnostic tool to distinguish p53 wild-type pregmalignant neoplasia from reactive vulvar lesions.

We acknowledge several limitations of our study. Due to its retrospective nature, much of the clinical information was unavailable. Parameters such as focality and exact localization could have influenced the results. In the study period (1991-2011), surgery was the preferred treatment method and imiquimod and laser vaporization were not routinely used, which does not adequately reflect current treatment regimes. Moreover, many patients received several additional treatments after baseline, which undoubtedly influences possible progression of disease. Although future prospective validation is needed, our findings suggest that methylation may play an important role in risk-adapted decisions on treatment and follow-up in patients with HSIL. In this regard it would be interesting to carry out a prospective study on the correlation between methylation and persistence of high-grade VIN, adjusted for type of treatment (i.e. imiquimod versus surgery). As our understanding of methylation continues to evolve with future, larger studies on patients with p53 wild-type dVIN, the potential implications for treatment and disease outcome will become clearer.

Our study also has multiple strengths. This is the first study demonstrating that positive methylation status was associated with progression to cancer in a large, population-based, longitudinal cohort series of 624 patients with high-grade VIN. This series was comprehensively characterized with revision and the use of IHC staining of $p16^{INK4a}$, p53 and ki-67, and HPV status. As described previously, accurate categorization of the type of VIN is essential for cancer risk assessment.[5] Long-term follow-up data was available with a median follow-up period of 17.4 years, making this a unique series of a rare disease.

In conclusion, this study shows that methylation status provides objective information on cancer risk and can therefore guide personalized treatment and monitoring of patients with HSIL. Patients with methylation-negative HSIL can be reassured of their low cancer risk and safely treated with a non-radical treatment modality, which will reduce morbidity and increase quality of life. For patients with dVIN, it is essential to determine p53 status as it is highly correlated with short-term cancer risk.

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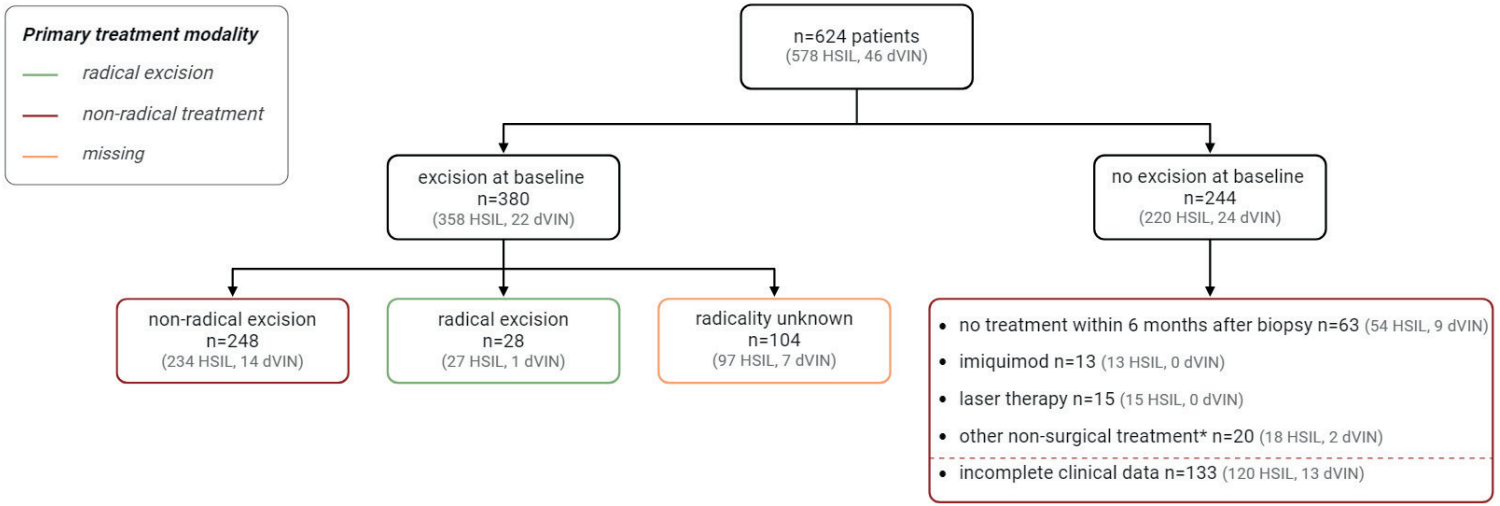
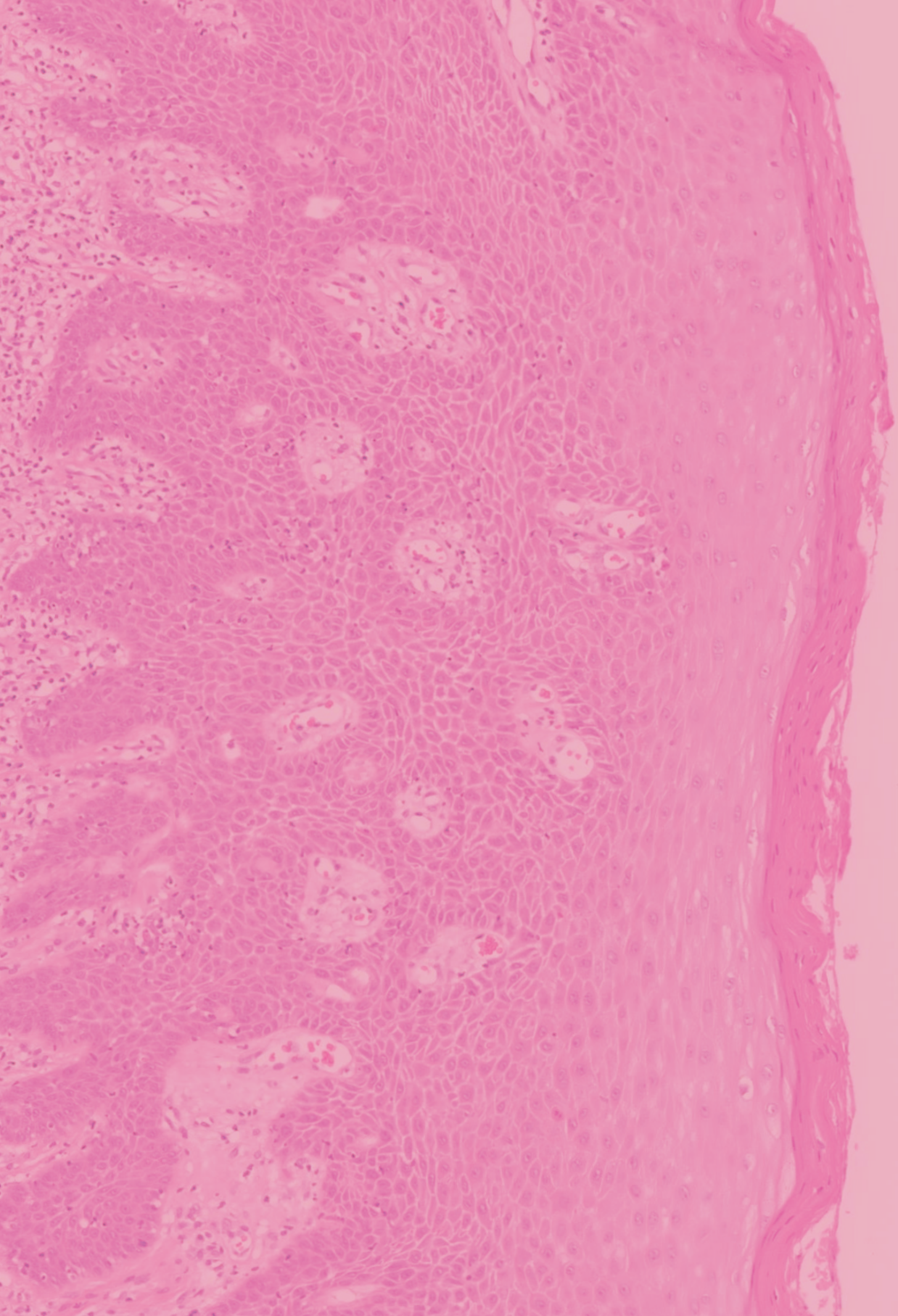


Supplementary Figure S1. Overview of the primary treatment modality of HSIL and dVIN patients within 6 months after initial biopsy.

* Patients who received other non-surgical treatment (18 HSIL and 2 dVIN) included either cryotherapy, electrocoagulation or topical treatment with either efudix, clobetasol proprionate, acitretin, hydrocortisone/miconazole, fusidic acid or econazole nitrate.



Chapter 9

Expression of CK17 and SOX2 in vulvar intraepithelial neoplasia: a comprehensive analysis of 150 vulvar lesions

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Abstract

Background: Recently, the immunohistochemical markers cytokeratin 17 (CK17) and SRY-box2 (SOX2) have been evaluated as adjuncts for the diagnosis of high-grade vulvar intraepithelial neoplasia (VIN). In the present study, the aim was to assess CK17 and SOX2 expression in VIN by studying 150 vulvar lesions, originally reported as high-grade VIN and to assess the diagnostic accuracy. Methods: All slides (H&E, $p16^{INK4a}$, p53, Ki-67, CK17, and SOX2 stains) were independently assessed by six pathologists and the final diagnosis was reached in consensus meetings, as follows: 46 human papillomavirus (HPV)-independent VIN (including 30 p53 mutant and 16 p53 wild-type lesions), 58 high-grade squamous intraepithelial lesions (HSILs), 4 low-grade SILs (LSILs), 37 non-dysplastic lesions, and 5 lesions where the histology was inconclusive. Results: CK17 positivity was observed in 100% p53 wild-type HPV-independent VIN, compared to 73% p53 mutant HPV-independent VIN, 14% HSILs, 0% LSILs, and 24% non-dysplastic lesions. SOX2 positivity was observed in 13% p53 wild-type HPV-independent VIN, 43% p53 mutant HPV-independent VIN, 2% HSILs, 0% LSILs, and 3% non-dysplastic lesions. The highest diagnostic accuracy (89%) for HPV-independent VIN was obtained when combining p53 and CK17 immunohistochemistry. The addition of SOX2 did not further increase diagnostic accuracy. Conclusion: To conclude, aside from p53, both CK17 and SOX2 can be of value for reaching an accurate diagnosis of HPV-independent VIN.

Introduction

High-grade vulvar intraepithelial neoplasia (VIN) is subdivided into human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesions (HSILs) and HPV-independent VIN, with the latter usually clinically referred to as differentiated VIN (dVIN) based on the WHO 2020 classification of female genital tumors [1]. Recent studies proposed the further subdivision of HPV-independent VIN into the following two prognostically significant groups: p53 mutant and p53 wild-type lesions [2,3]. Histopathological variants of p53 wild-type HPV-independent VIN include vulvar acanthosis with altered differentiation (VAAD), differentiated exophytic vulvar intraepithelial lesion (DEVIL), and verruciform lichen simplex chronicus, which have all recently been described and grouped under the term verruciform acanthotic VIN (vaVIN) [4]. The International Society of the Study of Vulvovaginal Diseases (ISSVD) recognizes the term vulvar aberrant maturation (VAM) to include DEVIL, VAAD, and other related p53 wild-type lesions [5]. Given the low incidence of HPV-independent VIN, the poor reproducibility and overlapping morphology, the terms VAAD, DEVIL, vaVIN, and VAM are not consistently applied. More importantly, this morphological subtyping does not reflect the biological nature in terms of cancer risk, and thus the usefulness of these terms in clinical practice can be questioned.

An accurate diagnosis of high-grade VIN and the distinction between HSILs and HPV-independent VIN are important, considering the implications for treatment and prognosis [2,6]. The 10-year risk of progression to invasive cancer is 67% for p53 mutant HPV-independent VIN, whereas this is 28 to 37% for p53 wild-type HPV-independent VIN, DEVIL, VAAD, and verruciform lichen simplex chronicus, and 6% in HSILs [2,7]. However, the diagnosis of HPV-independent VIN with a p53 wild-type staining pattern is particularly challenging [8]. Owing to limited cytonuclear atypia and overlapping histomorphological features, p53 wild-type HPV-independent VIN may be misdiagnosed as a non-dysplastic or reactive lesion, or as a low-grade squamous intraepithelial lesion (LSIL). Hence, there is a need for additional diagnostic markers to help differentiate p53 mutant HPV-independent VIN from its mimics.

Few studies have shown the potential value of the immunohistochemical markers CK17 and SOX2 in VIN [9–14]. Dasgupta et al. observed CK17- and SOX2-positive immunohistochemical staining in 81% and 86% of HPV-independent VIN and in 63% and 88% of HSILs, respectively, as compared to 9% and 20% of non-dysplastic lesions [10].

Cytokeratin 17 (CK17) is an intermediate filament protein that is induced in activated keratinocytes [15,16]. Previous investigations have demonstrated elevated CK17

expression in premalignant and malignant tissues compared to healthy tissues [17]. Sex-determining region Y-box 2 (SOX2) is located on chromosomal segment 3q26.33, serves as a critical regulator of pluripotent stem cells, and helps to maintain and develop the squamous epithelium [18]. Previous studies have indicated that SOX2 functions as an oncogene and is subject to highly recurrent genomic amplification in squamous cell carcinomas, including those of the anogenital region, lung, head and neck, and oral cavity [19,20].

The current study aimed to assess the expression rates of CK17 and SOX2 in VIN, as well as the contribution to diagnostic accuracy for the diagnosis of HPV-independent VIN.

Materials and Methods

Study Population and Categorization of Vulvar Lesions

From a population-based historical cohort consisting of 751 patients, all originally diagnosed with high-grade VIN, a subset of 150 cases were selected for the current study [2,21]. All 751 cases had previously been reviewed and, for the current study, all the cases of HPV-independent VIN, non-dysplastic lesions, and cases with inconclusive histology were selected, along with all the HPV-associated cases that were present on the slides used for immunohistochemistry of the aforementioned cases [2].

All 150 patients in the current cohort had no vulvar cancer history and no concurrent vulvar cancer at the time of high-grade VIN diagnosis. The HPV DNA test result was available for each case. Follow-up information on the progression to vulvar cancer was available from the nationwide registry of histopathology in the Netherlands [22]. Selected cases included part of the HPV-associated lesions and all HPV-independent lesions (i.e., HPV-independent VIN and non-dysplastic lesions), as well as all inconclusive cases, as concluded after a previous histopathological revision [2].

Using a data collection form and the PathXL online viewer, H&E and immunohistochemical slides were scored independently by five pathologists and one resident in pathology (N.B.T., K.V.D.V., P.C.E.G., S.D., J.B., and M.C.G.B), all with a high exposure to vulvar pathology. The final diagnosis was based on H&E, $p16^{INK4a}$, p53, Ki-67, and HPV results. Vulvar lesions were categorized as HPV-associated (HSIL or LSIL), HPV-independent VIN, or non-dysplastic lesions, such as lichen sclerosus (LS), reactive

lesions, and other non-dysplastic dermatoses. Discrepancies in the final diagnosis or immunohistochemical staining patterns were discussed in consensus meetings.

Tissue Processing

Details of the tissue processing, immunohistochemistry of $p16^{INK4a}$, p53, and Ki-67, DNA isolation, and HPV DNA testing have been described previously [2]. Immunostaining for CK17 and SOX2 was performed with the Optiview detection kit, with the automated 100 BenchMark ULTRA IHC/ISH system (Roche) and with mouse monoclonal antibodies against the keratin17 antigen (clone SP95; Abcam, Cambridge, UK) and the SOX2 antigen (clone EP103; Cellmarque, Rocklin, CA, USA).

Immunohistochemical Staining

Patterns for $p16^{INK4a}$, p53, Ki-67, CK1, and SOX2 $P16^{INK4a}$ staining was scored as negative (absent or patchy) or block (diffuse) positive (≤1/3, ≤2/3, >2/3). P53 staining was scored as wild type (scattered or mid-epithelial with basal sparing) or mutant (nuclear positive, null or cytoplasmic positive). Ki-67 staining was scored as not increased (a few positive parabasal nuclei) or increased (≤1/3, ≤2/3, >2/3) [2]. CK17 cytoplasmic staining was scored in the horizontal direction as patchy (50%), and SOX2 nuclear staining as scattered (50%) [10]. For both CK17 and SOX2, staining in the vertical direction was scored as either full-epithelial or partial thickness. Staining intensity was recorded as mild, moderate, or strong. Subsequently, for purposes of statistical analysis, the features of CK17 and SOX2 staining described above were combined, resulting in the following two final categories, similar to those used previously by Dasgupta et al: negative (no expression, patchy or scattered staining, or staining with weak intensity) or positive (diffuse and moderate-to-strong intensity) (Figure 1) [10].

Statistical Analysis

Immunohistochemistry scores for each disease category were compared using Pearson's Chi-Squared test. The level of statistical significance was set at 0.05. The performance of p53, CK17, and SOX2 immunohistochemical markers was calculated for the diagnosis of HPV-independent VIN using non-dysplastic lesions as a control group. This included sensitivity, specificity, and accuracy calculations. These calculations included a 95% confidence interval (95% CI). Statistical analysis was performed using IBM SPSS Statistics software for Windows version 28.0 (IBM Corporation, Armonk, NY, USA).

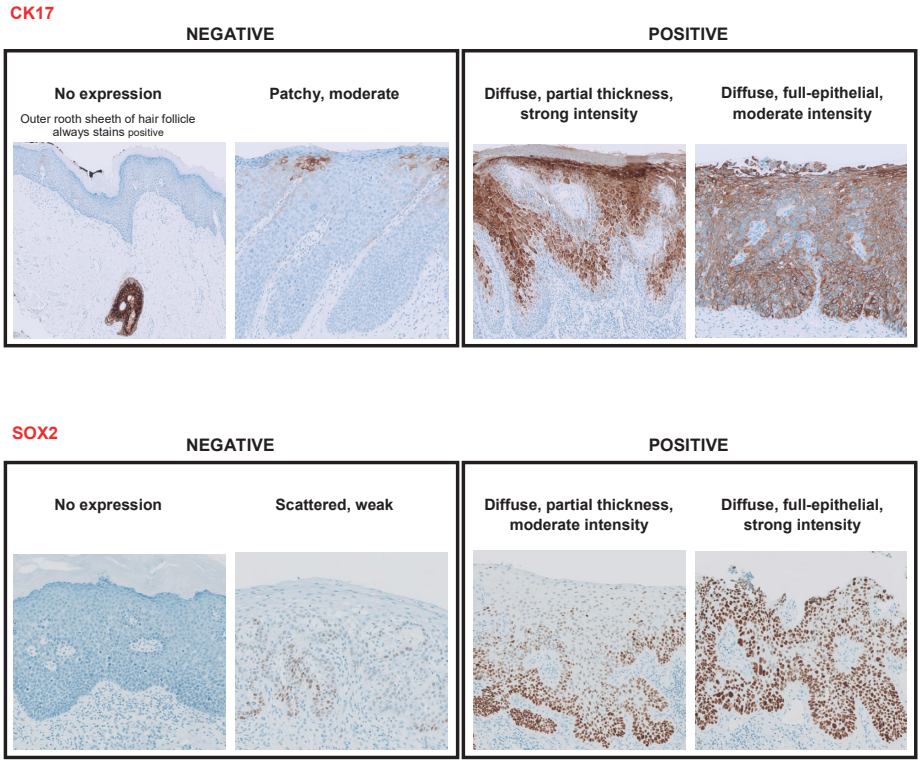

Figure 1. Representative examples of CK17 and SOX2 immunohistochemical staining patterns. CK17 and SOX2 staining patterns were categorized as negative (no expression, patchy or scattered staining, or staining with weak intensity) or positive (diffuse and moderate to strong intensity). CK17 is positive in the outer root sheath of the hair follicle epithelium.

Results

Vulvar Disease Categories

After revision of the H&E images and immunohistochemistry by the participating pathologists, consensus was reached on the diagnoses for the study cohort, which comprised 46 HPV-independent VIN (30 p53 mutant and 16 p53 wild-type), 58 HSILs, 4 LSILs, 37 non-dysplastic lesions, and 5 inconclusive lesions. Non-dysplastic lesions included LS (n = 7), inflammation (n = 11), reactive changes (n = 12), (fibro-)epithelial polyps (n = 2), and vulvar tissue without histological abnormalities (n = 5).

The immunohistochemical expression of the markers in relation to the final diagnoses are shown in Table 1.

Block-positive p16^{INK4a} was observed in all the HSILs (100%) and in 1/46 (3%) of the HPV-independent VIN ($p < 0.001$). The single p16^{INK4a} block-positive HPV-independent VIN demonstrated p53 mutant staining and tested negative for HPV. Mutant p53 staining was observed in 30/46 (65.2%) of the HPV-independent VIN. One lesion in this series did not meet the criteria for HPV-independent VIN based on histomorphology but did demonstrate a p53 mutant staining pattern. This case was categorized as inconclusive. None of the 37 non-dysplastic lesions displayed mutant p53 staining.

CK17 Immunohistochemistry

CK17 expression patterns differed significantly across all disease categories ($p < 0.001$) (Table 1). Positive CK17 staining (i.e., a diffuse, moderate-to-strong staining pattern) was seen in 38/46 (83%) of the HPV-independent VIN, including 22/30 (73%) of the p53 mutant HPV-independent VIN and all 16 (100%) of the p53 wild-type HPV-independent VIN. In the HSIL, LSIL, and the non-dysplastic lesions, lower CK17 positivity rates of 14%, 0%, and 24% were observed, respectively.

Of the 38 CK17-positive cases of HPV-independent VIN, 63% showed full-epithelial CK17 expression. CK17 staining was often seen in the more superficial keratinocytes showing differentiation. Representative examples of immunohistochemical staining patterns in HPV-independent VIN are shown in Figures 2 and 3.

The CK17-positive non-dysplastic lesions ($n = 9$) included three cases of LS, two reactive lesions, two cases of hyperplasia, and two epithelial polyps. The CK17-negative non-dysplastic lesions ($n = 27$) included 11 lesions showing inflammation, 4 cases of LS, 10 reactive lesions, and all 5 normal epithelia. The p53 mutant inconclusive case showed an absence of CK17 staining.

SOX2 Immunohistochemistry

SOX2 expression patterns also differed significantly across all disease categories ($p < 0.001$) (Table 1). Positive SOX2 staining was seen in 15/46 (33%) of the HPV-independent VIN; in 13/30 (46%) of the p53 mutant HPV-independent VIN and in 2/16 (13%) of the p53 wild-type HPV-independent VIN ($p = 0.023$). The majority of HSIL, LSIL and non-dysplastic cases showed negative staining for SOX2. Only 2% of the HSILs showed positive staining, while none of the LSIL cases did so. In addition, only one non-dysplastic (reactive) lesion stained positive for SOX2. SOX2 was negative in the five vulvar epithelia without abnormalities.

Table 1. Immunohistochemical expression of p53, CK17, SOX2 p16^{INK4a}, and ki-67 in relation to different categories of vulvar lesions.

IHC marker	Expression pattern	HPV-independent VIN				HPV-associated SIL				Non-dysplastic		Inconclusive	
		p53 mutant		p53 wild-type		HSIL		LSIL					
		n=30	(%)	n=16	(%)	n=58	(%)	n=4	(%)	n=37	(%)	n=5	(%)
P53	Wild-type	0	(0)	16	(100)	58	(100)	4	(100)	37	(100)	4	(80)
	Mutant	30	(100)	0	(0)	0	(0)	0	(0)	0	(0)	1	(20)
CK17	Negative	8	(27)	0	(0)	50	(86)	4	(100)	27	(73)	2	(40)
	No expression	0	(0)	0	(0)	16	(28)	2	(50)	17	(46)	1	(20)
	Patchy	8	(27)	0	(0)	34	(59)	2	(50)	10	(27)	1	(20)
	Positive	22	(73)	16	(100)	8	(14)	0	(0)	9	(24)	3	(60)
	Full-epithelial	8	(27)	6	(38)	1	(2)	0	(0)	3	(8)	1	(20)
	Partial thickness	14	(47)	10	(63)	7	(12)	0	(0)	6	(16)	2	(40)
	Not judgeable	0	(0)	0	(0)	0	(0)	0	(0)	1	(3)	0	(0)
SOX2	Negative	17		14		57		4	(100)	35	(95)	5	(100)
	No expression	11	(37)	9	(56)	53	(91)	3	(75)	24	(65)	2	(40)
	Patchy	6	(20)	5	(31)	4	(7)	1	(25)	11	(30)	3	(60)
	Positive	13	(43)	2	(13)	1	(2)	0	(0)	1	(3)	0	(0)
	Full-epithelial	8	(27)	0	(0)	1	(2)	0	(0)	0	(0)	0	(0)
	Partial thickness	5	(17)	2	(13)	0	(0)	0	(0)	1	(3)	0	(0)
	Not judgeable	0	(0)	0	(0)	0	(0)	0	(0)	1	(3)	0	(0)

IHC marker	Expression pattern	HPV-independent VIN				HPV-associated SIL				Non-dysplastic		Inconclusive	
		p53 mutant		p53 wild-type		HSIL		LSIL					
		n=30	(%)	n=16	(%)	n=58	(%)	n=4	(%)	n=37	(%)	n=5	(%)
P16INK4a	Negative	29	(97)	16	(100)	0	(0)	3	(75)	36	(97)	5	(100)
	Block-positive	1	(3)	0	(0)	58	(100)	1	(25)	0	(0)	0	(0)
	Not judgeable	0	(0)	0	(0)	0	(0)	0	(0)	1	(3)	0	(0)
Ki-67	Not increased	4	(13)	2	(13)	0	(0)	0	(0)	20	(54)	1	(20)
	Increased	26	(87)	14	(88)	58	(100)	4	(100)	17	(46)	4	(80)

Of all the SOX2-positive HPV-independent VIN, eight cases (53%) showed SOX2 expression across the full-epithelial thickness (Figure 2), and seven cases (47%) showed partial thickness staining (Figure 3).

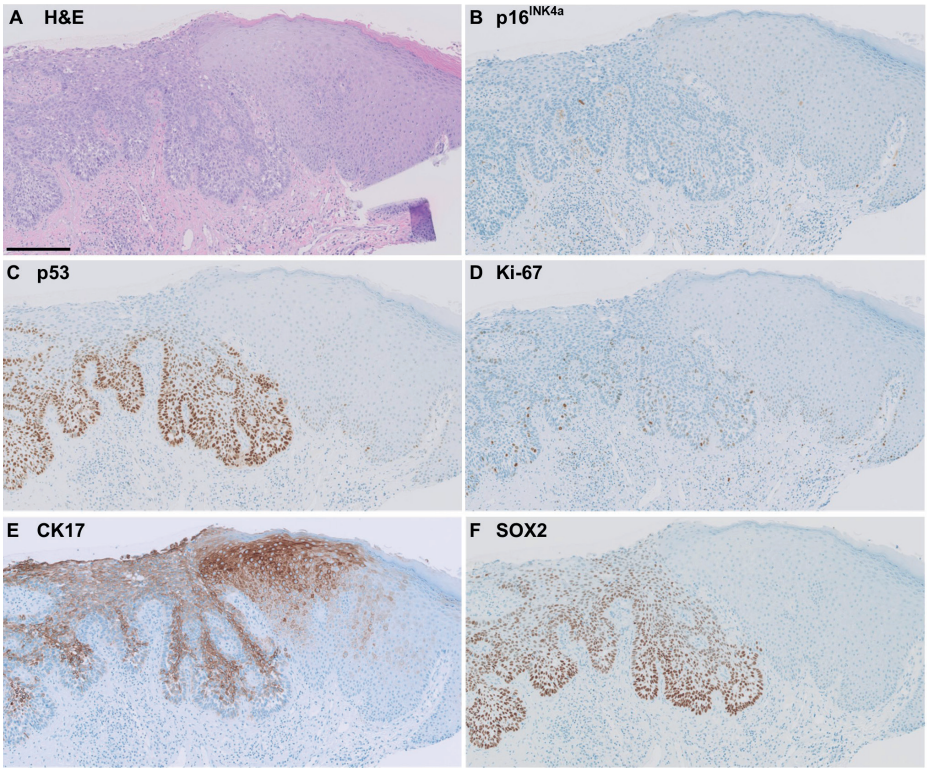

Figure 2. Example of immunohistochemical staining pattern observed in p53-mutant HPV-independent VIN, showing classical 'differentiated' features. Bar = 200 µm. (A) H&E staining. (B) Negative $p16^{INK4a}$. (C) positive p53 staining – mutant pattern. (D) No increased Ki-67. (E) Positive CK17, diffuse, moderate intensity and near full-epithelial expression. (F) Positive SOX2, diffuse, moderate to strong intensity, full-epithelial expression.

Performance of Markers for Accurate Diagnosis of HPV-Independent VIN

Significantly more CK17 and SOX2 positivity was observed in HPV-independent VIN compared to non-dysplastic cases ($p < 0.001$). The performance of p53, CK17, and SOX2 in relation to the diagnosis of HPV-independent VIN is shown in Table 2. P53 and CK17 had a high accuracy for the detection of HPV-independent VIN (80% and 79%, respectively). Although SOX2 showed a high specificity of 97%, the low sensitivity of 33% resulted in a moderate accuracy for the detection of HPV-independent VIN of 61%.

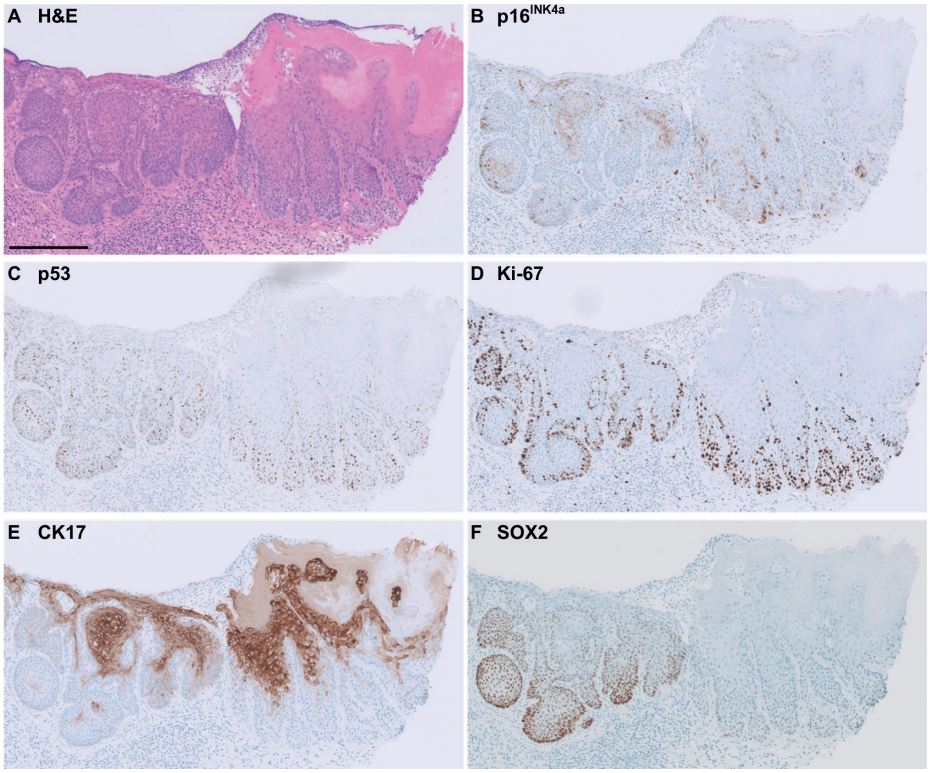

Figure 3. Example of immunohistochemical staining pattern observed in p53 wild-type HPV-independent VIN. Bar = 200 µm. (A) H&E staining. (B) Negative (patchy) $p16^{INK4a}$. (C) Wild-type pattern of p53 staining. (D) Increased Ki-67 expression up to 1/3rd of epithelial thickness. (E) Positive CK17, diffuse, moderate-strong intensity and partial thickness expression. (F) Positive SOX2, diffuse, moderate-strong intensity, partial thickness expression.

Combining the markers p53 and CK17 - in other words, where cases had to show either mutant p53 staining and/or positive CK17 staining - resulted in the highest accuracy for the diagnosis of HPV-independent VIN of 89%. Adding SOX2 to this combination did not further increase accuracy.

Prognostic Value of CK17 and SOX2

Of the 36 non-dysplastic lesions, 13 (35%) progressed to VIN during follow-up. Of these, five (42%) were positive with CK17 and one (8%) was SOX2 positive. Of the 24 non-dysplastic cases that did not progress to VIN, 4 (17%) were CK17 positive and none were SOX2 positive. Hence, a significant correlation between CK17 and SOX2 staining and progression to dysplasia could not be demonstrated ($p = 0.102$ and $p = 0.151$, respectively).

Within the total follow-up period of 23.3 years, 25/46 (54%) HPV-independent VIN progressed to vulvar cancer. Of the 14 cases that progressed to vulvar cancer within two years, 12 (86%) and 7 (50%) were found to be CK17 and SOX2 positive, respectively. Of the 32 cases that did not progress to cancer within two years, 26 (81%) and 8 (25%) were CK17 and SOX2 positive, respectively. A significant correlation between CK17 and SOX2 staining and progression to vulvar cancer within two years could not be demonstrated (p = 0.713 and p = 0.096, respectively).

Table 2. Test characteristics of p53, CK17, and SOX2 immunohistochemistry in HPV-independent VIN. Non-dysplastic lesions with a valid immunohistochemical result were used as control group. NB: not corrected for disease prevalence.

		Sensitivity		**Specificity**		**Accuracy**	
HPV-independent VIN	**IHC marker**	**% (95% CI)**		**% (95% CI)**		**% (95% CI)**	
All	P53	65	(50-79)	100	(90-100)	80	(71-89)
n=46	CK17	83	(69-92)	75	(58-88)	79	(69-87)
	SOX2	33	(20-48)	97	(85-99.9)	61	(70-88)
	P53/CK17	100	(92-100)	76	(59-88)	89	(80-95)
	P53/SOX2	70	(54-82)	95	(82-99)	81	(71-89)
	P53/CK17/SOX2	100	(92-100)	70	(53-84)	87	(78-93)
P53 mutant	CK17	73	(54-88)	75	(58-88)	74	(62-84)
n=30	SOX2	43	(26-63)	97	(85-99.9)	73	(60-83)
P53 wild-type	CK17	100	(79-100)	75	(58-88)	83	(70-92)
n=16	SOX2	13	(2-38)	97	(85-99.9)	71	(57-83)

Discussion

Our study demonstrated positive CK17 staining (i.e., a diffuse, moderate-to-strong staining pattern) in the majority of HPV-independent VIN (83%), with 73% in p53 mutant HPV-independent VIN and 100% in p53 wild-type HPV-independent VIN. This is consistent with the findings of others [11,12,14]. CK17 expression in VIN was first described in 2017, showing increased expression in up to 93% of HPV-independent VIN [14]. More recently, other research groups have reported an increased CK17 expression in HPV-independent VIN (81 to 100%), which is similar to our study. SOX2 demonstrated significantly more positive staining in p53 mutant HPV-independent VIN than in p53 wild-type HPV-independent VIN but showed

lower accuracy for the diagnosis of HPV-independent VIN than CK17, owing to low sensitivity. Combining p53 and CK17 showed the highest accuracy for the diagnosis of HPV-independent VIN. The addition of SOX2 did not further increase the accuracy.

There is a clinical need for additional diagnostic markers for the accurate diagnosis of p53 wild-type HPV-independent VIN, given that the prognosis differs substantially from its mimickers [2]. Several studies have reported on CK17 expression in relation to p53 staining patterns in HPV-independent VIN [11–14]. Such stratified data were also provided to us by Dasgupta et al. [10]. Interestingly, when combining all p53 wild-type HPV-independent VIN cases from the four aforementioned studies, 57/62 (92%) of the cases showed positive CK17 expression, comparable to the 100% positivity rate observed in this series. When combining all p53 mutant HPV-independent VIN cases from these studies, 72/81 (89%) of the cases demonstrated positive CK17 expression, which is a little higher than the 73% found in our study. One possible explanation for this difference is the varying proportion of cases with concurrent vulvar cancer between these studies.

CK17 exhibits a diffuse and moderate-to-strong expression in most cases of p53 wild-type HPV-independent VIN. In particular, when combined with p53, the specificity of CK17 is high, i.e., if CK17 is negative in a p53 wild-type lesion, this argues against an HPV-independent VIN. This information can aid a pathologist in making an accurate diagnosis and may even potentially be of use for the assessment of surgical margins. Studies have shown that patients with HPV-independent VIN in the resection margin have a poorer prognosis [23,24].

Evaluating the CK17 staining patterns, it was observed that in most cases, there was an absence of staining in the basal layers. These CK17-negative basal cell layers often had scant cytoplasm and a small nucleus, consistent with 'basal-like' histomorphology. The significance of this pattern is unclear, but one hypothesis is that full-epithelial CK17 expression is increasingly seen in epithelia with greater invasive potential as CK17 can promote propagation and inhibit apoptosis of cells [25]. In squamous cell carcinoma of the anus, esophagus, and oral cavity, CK17 is thought to be associated with disease progression [26–28]. Changes in the expression of CK17 that may occur during disease progression may be relevant to establish a prognosis. However, in the current study, a correlation between full thickness CK17 staining and invasive potential could not be demonstrated. The low sample size and influence of subsequent treatment of HPV-independent VIN may be of relevance here.

Despite having used the same scoring methodology as Dasgupta et al. [9,10] in our study, the sensitivity of SOX2 for the diagnosis of HPV-independent VIN was found to be much lower. Also of note is the fact that CK17 and SOX2 were positive in only 14% and 2% of the HSIL cases, respectively, much lower than has previously been reported [20]. Several potential explanations exist for this disparity. Firstly, different clones were used for immunohistochemical staining. Secondly, in our series, no cases adjacent to carcinoma were selected, while this proportion was 52% in the study of Dasgupta.

CK17 and SOX2 displayed a greater specificity for HPV-independent VIN when used in conjunction with p53. Some mimickers of p53 wild-type HPV-independent VIN, such as LS, also frequently showed positive CK17 staining, making CK17 less discriminative between these cases. Positive CK17 in LS was observed in 43% of the cases in this study, compared to in 60 to 90% in the previous reports [11–14]. The relatively high positivity rate for CK17 in LS is especially problematic when part of the LS epithelium displaying positive CK17 is suspicious for HPV-independent VIN. In our series, all seven LS cases were SOX2 negative, and thus SOX2 could potentially differentiate between CK17-positive LS and CK17-positive HPV-independent VIN. Cook et al. also found no positive SOX2 expression ('score 3+') in four LS cases [11]. However, in contrast to these findings, one other study found SOX2 to be negative in only 44% (4/9) of the LS cases [29]. It should be noted that the aforementioned studies, including ours, had a low number of LS cases, making it difficult to draw reliable conclusions. Although our results did not show a high sensitivity for SOX2 as an individual marker, its specificity was high. SOX2, as part of a panel with other stains, may be of additional value in the differential diagnosis between p53 wild-type HPV-independent VIN and (atypical) LS.

The main strength of this study is that it includes a large series of 150 vulvar lesions which were independently assessed by six pathologists. All cases were without concurrent vulvar cancer and selected from a well-defined population-based cohort, reducing the risk of selection bias. The large variety of vulvar lesions in this series, including both HPV-associated and HPV-independent lesions as well as inconclusive cases, provided us with the opportunity to comprehensively assess the markers as initially evaluated by Dasgupta et al. [10]. All 150 cases were stained with the five immunohistochemical markers and agreement was achieved for all diagnoses and interpretations of immunohistochemical stains through multiple consensus meetings.

Our study also has several limitations. Due to the retrospective study design, no clinical data were available when reviewing cases, a limitation that may be particularly important for the optimal interpretation of difficult cases. In addition, only a small subset of the non-dysplastic lesions were cases of LS, while this is amongst the most important differential diagnoses. Additional series including non-dysplastic mimickers of HPV-independent VIN are needed to further explore the diagnostic accuracy of the markers tested here. Although we used the same quantitative cut-offs as Dasgupta et al., a uniform and standardized scoring system for CK17 and SOX2 is not yet available [10]. On the other hand, immunohistochemistry is an ideal technique to evaluate biomarker expression because it is fast, easy, and relatively cheap.

Conclusions

This study assessed the values of CK17 and SOX2 immunohistochemistry as adjunct diagnostic markers for the accurate diagnosis of HPV-independent VIN. When used in combination with p16 and p53, CK17, in particular, can aid a correct diagnosis. SOX2 can be particularly helpful when there is a differential diagnosis between HPV-independent VIN and LS, but larger studies with more LS cases are needed to confirm this.

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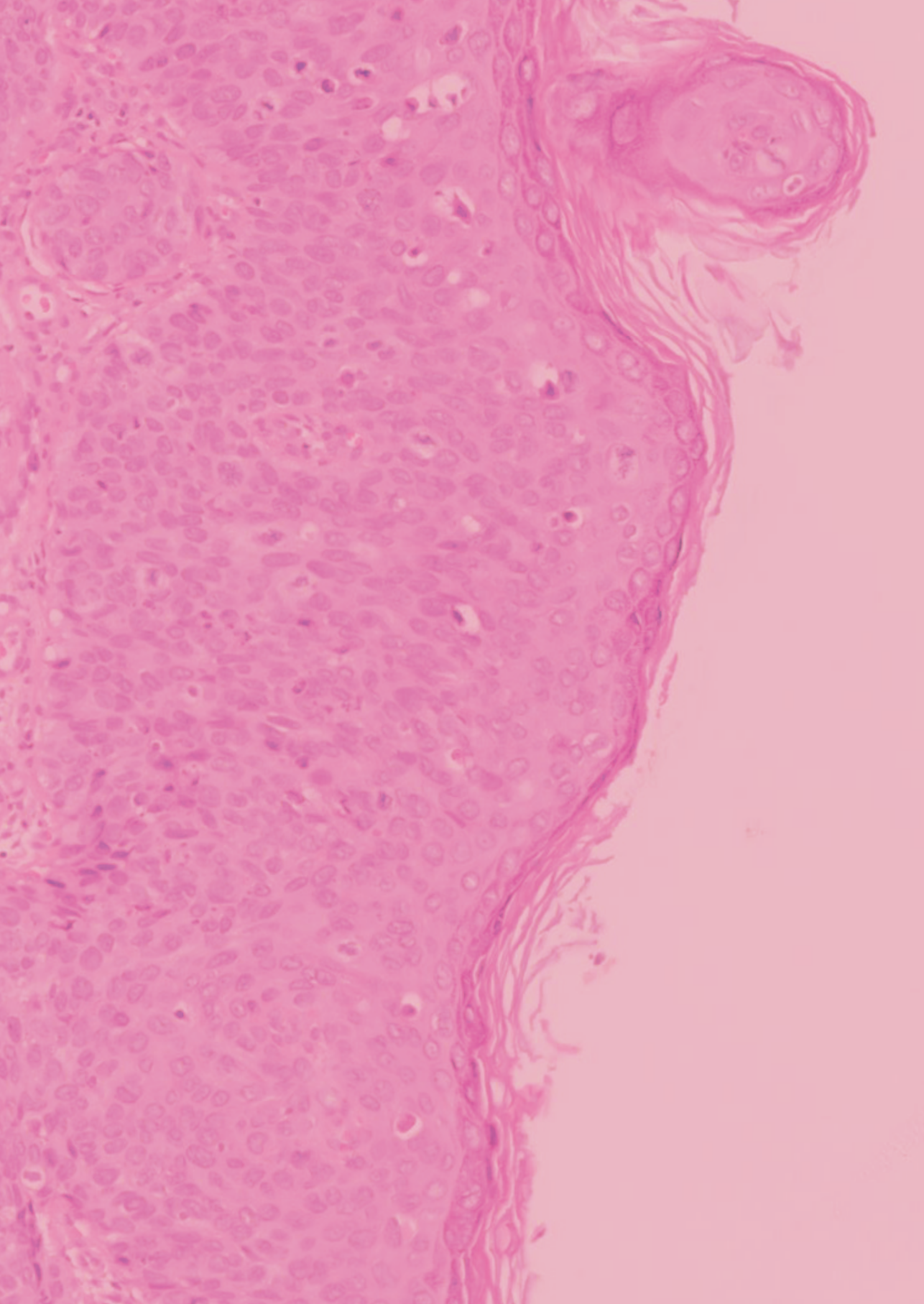

CHAPTER 10

General discussion and future perspectives

The research described in this thesis investigates the potential risk factors for progression to cancer in high-grade vulvar intraepithelial neoplasia (VIN) patients. Most of this research is based on data that we have generated from a large, population-based, historical Dutch cohort of patients, originally diagnosed with high-grade VIN between 1991 and 2011. These patients were diagnosed in the region of Amsterdam, including the three academic medical centers and their affiliated referral centers, which in total comprised 18% of the female population in the Netherlands at the time. From this historical cohort, we have extensively studied VIN lesions diagnosed at time of first presentation, both human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesions (HSIL) and HPV-independent VIN, the latter also known as differentiated VIN (dVIN). We have investigated the diagnostic and prognostic potential of several biomarkers, including DNA methylation markers of twelve genes, HPV DNA genotyping, and immunohistochemical (IHC) expression patterns. Other potential risk factors for progression to vulvar cancer included age and presence of lichen sclerosus (LS).

In this chapter, the results of our studies are discussed in the perspective of current literature, possible clinical implications and future directions.

Towards a clinically relevant classification of VIN

Although the presence of two types of VIN has been known since the 1960s, the relevance of this distinction has only come to greater attention in the past ten to fifteen years. This is evidenced by the substantial increase in published literature in this area since then.(1-9) The study in **Chapter 2** presents a large population-based historical cohort of 894 patients, without previous or concurrent vulvar cancer, diagnosed with high-grade VIN between 1991 and 2011. In this historical cohort, only 12 cases had been reported as HPV-independent VIN (dVIN), while the remainder of cases was considered HSIL. At that time, VIN was usually only categorized based on the degree of dysplasia (i.e. VIN1, VIN2 or VIN3) rather than by etiology.(10) Although clear clinical differences were observed between the two patient groups harboring two different types of VIN, only a few studies had addressed these clinical differences.(11-13) Moreover, as imiquimod and laser treatments were not yet widely in use, and both VIN types were similarly treated by surgery, histopathological distinction between the two types of VIN was clinically less relevant. As a result, the less common HPV-independent VIN subtype was underreported. Several other factors have also contributed to the low incidence of HPV-independent VIN.(14-16) Firstly, HPV-independent VIN is often poorly recognized due to challenging clinical and pathological characteristics overlapping with other vulvar conditions.(14-17) Secondly, HPV-independent VIN mainly affects older patients, who may delay seeking

medical care.(18) Diagnostic delays, coupled with the aggressive course of HPV-independent VIN, often results in late-stage and missed diagnoses, and progression to vulvar cancer.(19, 20) In the historical cohort, 63% of HPV-independent VIN were diagnosed when progression to vulvar cancer had already occurred. In addition, the majority of vulvar cancers are HPV-independent, from which it can be concluded that its precursor, HPV-independent VIN, is insufficiently recognized.(**Chapter 2**).(15)

Most published series on VIN are single center-based and only include selected cases.(19, 21) The Nationwide network and registry of histopathology and cytopathology (PALGA) in The Netherlands is unique in this regard, making it possible to investigate large numbers of rare diseases, such as VIN.(22) By combining data from PALGA and Statistics Netherlands we were able to calculate the incidence of HSIL and HPV-independent VIN between 1991 and 2011 (**Chapter 2)**.(22, 23) As was also shown by others, the incidence of HSIL increased during the study period.(15, 20, 24) Several factors have contributed to this rise, including increased rates of HPV-infection, increased proportion of patients with immune deficiency disorders, and increased awareness of vulvar predisposing diseases.(25) The increased incidence of HPV-independent VIN in the last decades can be explained by the increased incidence of LS, more frequent use of IHC staining by pathologists, higher life expectancy of patients, and increased awareness of the disease among patients, clinicians and pathologists.(20, 26, 27)

In our historical cohort (**Chapter 2**) the vulvar cancer risk after three years was 43% in HPV-independent VIN and 6% in HSIL.(15) Despite the small number of HPV-independent VIN, this study was one of the largest population-based, longitudinal series ever published. Van de Nieuwenhof et al. had previously published a similar study, with comparable findings, including a vulvar cancer risk of 33% in HPV-independent VIN versus 6% in HSIL after a median time of three years.(15, 24) The lower cancer risk of HPV-independent VIN observed in this study compared to our study can be explained by the categorization of HSIL with LS as HPV-independent VIN. We have shown that the combination of HSIL and LS can indeed occur in a small proportion of HSIL patients, in our series in 2% of the HSIL patients.(15) Nevertheless, the high cancer risk of HPV-independent VIN as well as the short progression time to vulvar cancer has been described in several studies since then.(19, 20, 28)

In recent years, several studies have demonstrated that HPV-associated lesions and HPV-independent lesions can histomorphologically mimic each other.(29-32) Even though focal presence of HPV-independent VIN in a biopsy with predominantly 'HPV-associated-like' morphology is suggestive of the diagnosis of HPV-independent VIN, standard use of $p16^{INK4a}$ (surrogate marker for high-risk HPV infection) and p53

(marker reflecting an underlying *TP53* mutation, which is present in two thirds of HPV-independent VIN cases) IHC on every VIN biopsy is highly recommended, as shown in **Chapter 6** and by others.(16, 31) From 751 of 894 (84%) VIN cases of the historical cohort, we were able to retrieve pathology specimens for further evaluation. After histopathological re-evaluation, it was found that as many as 39/46 (85%) of HPV-independent VIN were originally not classified as such.(16) This can partly be explained by HPV-independent VIN displaying 'HPV-associated-like' histomorphology in 41% of the cases, while, on the other hand, low-grade squamous intraepithelial lesion (LSIL) may mimic HPV-independent VIN. This may also explain why in the first - histopathologically unrevised - study of the historical cohort, age and LS were found as risk factors for progression to vulvar cancer in patients with VIN. Those HSIL with LS cases (51.4%) were identified as HPV-independent VIN after reassessment of the cases, which has a more aggressive natural course than HSIL.(15, 16)

Accurate classification is of utmost importance given the varying cancer risks of HSIL and HPV-independent VIN. In recent years, efforts have been made to assign names to the various morphological subtypes of HPV-independent VIN, e.g. differentiated exophytic vulvar intraepithelial lesion (DEVIL), vulvar acanthosis with altered differentiation (VAAD), verruciform acanthotic VIN (VaVIN), and vulvar aberrant maturation (VAM).(33-37) Given the very low incidence, the poor reproducibility and the occurrence of an overlapping morphological spectrum, these terms are not widely applied. More importantly, this morphological classification does not reflect the natural course of the disease, thus fails to fulfill its clinical utility.(33, 34, 37, 38)

Recommendation of a three-tiered classification of VIN

After re-assessment and accurate categorization of all available VIN lesions of our historical cohort, it was demonstrated for the first time that cancer risk differs strikingly between i) HSIL, ii) p53 mutant HPV-independent VIN, and iii) p53 wild-type HPV-independent VIN, with 5-year cancer risks of 5%, 63% and 13%, respectively (**Chapter 6).**(16) Also, the median time to cancer was much longer in HSIL patients than in p53 mutant- and p53 wild-type HPV-independent VIN patients (6.0, 1.5, and 5.1 years, respectively). For vulvar cancer, the same three subcategories have been proposed by multiple studies. P53 mutant HPV-independent vulvar cancers had an increased recurrence risk and decreased overall- and disease-free survival compared to p53 wild-type carcinomas.(2, 20, 39-45) Given the important clinical consequences, we recommend this three-tiered categorization also for VIN.

Biomarkers in VIN

Immunohistochemical (IHC) markers

Several IHC markers are available for an accurate diagnosis of HPV-associated vulvar lesions. For HSIL the most important marker is $p16^{INK4a}$, which showed block-positivity in 98.9% of HSIL in our series.(46, 47) This high percentage corresponds to the $p16^{INK4a}$ proportion positives observed in other studies on VIN, and it is similar to the proportion positives at other anogenital sites such as the cervix and anus.(48, 49) Also, p53 IHC can contribute to a correct diagnosis, because the p53 wild-type pattern with increased mid-epithelial staining, in combination with a p53 wild-type scattered pattern of the basal cell layer, is only observed in HPV-associated lesions (in 48.6% of our series) and never in HPV-independent VIN.(16, 39, 50-53) A pitfall of this staining pattern is that it can mimic a p53 mutant pattern. A likely explanation for the increased mid-epithelial expression of p53 in HPV-induced lesions is the presence of an E6 splice variant that cannot degrade p53.(54, 55) Another caveat in assessing p53 in HPV-associated lesions is reduced p53 staining, sometimes mimicking a null-mutant pattern.(16, 51) The reduced expression of p53 in HPV-associated lesions is caused by the increased activity of the HPV E6 oncogene resulting in p53 degradation.(56, 57) Finally, we have shown that 'viral' Ki-67 staining, i.e. increased Ki-67 staining in the upper layers with less or no increased staining in the underlying layers, is helpful in making the diagnosis of HPV-associated lesions. This 'viral' Ki-67 pattern was seen in 39% LSIL and never in HPV-independent lesions.(16) To our best knowledge, 'viral' Ki-67 staining has only been referred to twice.(58, 59) If a 'viral' Ki-67 pattern is not recognized, proliferation-activity can easily be overestimated, which could erroneously lead to higher VIN grade and potentially overtreatment.

For the diagnosis of HPV-independent VIN, only a few IHC markers have utility in the diagnostic workup. In our studies, mutant p53 staining was demonstrated in 65% of HPV-independent VIN and virtually never in HPV-associated lesions, which corresponded to the numbers found in other reports.(16, 30) Increased Ki-67 staining was observed in 87% of HPV-independent VIN and in 46% of non-dysplastic lesions.

Several other IHC markers have been studied, including CK17, SOX2 and GATA3 (**Chapter 9**).(60-63) These studies indicated that if a p53 wild-type lesion has no CK17 and/or SOX2 expression, this argues against presence of HPV-independent VIN. Loss of GATA3 staining can help distinguish HPV-independent VIN from LS and HSIL, which both express moderate to strong GATA3 staining.(64) Additional validation studies should examine larger numbers of vulvar lesions, particularly those that are

included in the differential diagnosis of HPV-independent VIN, such as hyperplastic lesions or LS with varying degrees of atypia. Ideally, associated cancer risks should also be determined.

HPV testing

HPV testing can be useful when, despite the use of IHC, a lesion cannot be classified as either HPV-associated or HPV-independent. In our series, 99% of HPV-associated lesions had an HPV-positive result, compared to 11% of HPV-independent lesions, including 15% of HPV-independent VIN (**Chapter 6**).(16) Testing for the presence of high- and/or low-risk HPV DNA for correct classification can be helpful in $p16^{INK4a}$ negative/p53 wild-type lesions if either LSIL or p53 wild-type HPV-independent VIN are included in the differential diagnosis. Of note, detection of HPV DNA in a $p16^{INK4a}$-negative lesion does not necessarily prove a functional role of HPV.(16, 31) In HPV-independent VIN we found a higher rate of HPV positivity than anticipated based on overall HPV prevalence, particularly given that patients with HPV-independent VIN are generally older.(16) A likely explanation is prolonged use of topical corticosteroids in HPV-independent VIN patients with associated LS, which can counteract clearance by the immune system, or can reactivate a latent HPV infection.(65) For this reason, HPV infections are not uncommon in patients with LS.(32, 66, 67)

DNA methylation

Testing for DNA methylation of specific genes (methylation markers) could guide a correct diagnosis and predict clinical outcome when IHC and HPV testing fail to accurately categorize a vulvar lesion. The differential diagnosis in ambiguous cases often concerns $p16^{INK4a}$ negative/p53 wild-type lesions, or more specifically, p53 wild-type HPV-independent VIN versus either reactive lesions, or $p16^{INK4a}$-negative LSIL. In this thesis we have shown that DNA methylation levels increase with severity of vulvar disease, independent of VIN subtype.(68, 69) HPV-independent VIN showed positive methylation of the three-gene marker panel, consisting of *ZNF582, SST* and *miR124-2,* in nearly 90% of cases. In comparison, 36% of LSIL and 21% of reactive, non-dysplastic lesions demonstrated methylation-positivity. As these LSIL and non-dysplastic lesions in our study were at the time of original diagnosis classified as high-grade VIN, those percentages are possibly overestimated, and lower methylation-positivity rates are expected in routine diagnostics in these patient groups.(68) Only a few other reports have examined methylation in cross-sectional cohorts of VIN and vulvar cancer, with genes *CDKN2A* and *MGMT* being researched most often.(70-78) In these studies, *MGMT* methylation was detected in 45% (13/20) and 37% (11/30) of vulvar carcinomas, while in contrast, in our series 98% (57/58) of carcinomas exhibited high methylation levels. (74, 78) Interestingly, also the predictive value of methylation was recently objectified

in two studies, demonstrating that HPV DNA methylation could predict response to treatment with imiquimod in vulvar HSIL.(79, 80)

Cancer risk assessment in patients with VIN

HSIL

The prognostic value of methylation was first demonstrated in our historical cohort, in which methylation-positive HSIL displayed an almost 5-fold higher 5-year cancer risk compared to methylation-negative HSIL (**Chapter 8**).(81) These results support that the accumulation of epigenetic changes reflect progression of the carcinogenic process, as has also been demonstrated for cervical and anal HSIL, and genetic changes.(1, 69, 82-86) Of note, no studies other than ours have shown this for vulvar HSIL.

Once the diagnostic, prognostic and predictive value of methylation markers is validated in prospective studies, methylation tests can be used to guide management of VIN patients. We initiated in 2018 the **VENUS** (Vulvar intraEpithelial Neoplasia in sitU Study), a national, multicenter, prospective study, including patients with HSIL and HPV-independent VIN. The VENUS collects comprehensive clinicopathological data with long-term follow-up to further optimize systematic research into VIN. The study aims to include 300 VIN patients with at least 2 years of follow-up.

As none of 168 patients with methylation-negative HSIL progressed to cancer in the first four years of follow-up, and as in this group the median time to cancer was as long as 13 years, a negative methylation result would support conservative treatment. Imiquimod and laser, contrary to surgical excision, could help preserve anogenital structures (**Chapter 8)**.(87-89) HSIL patients with a methylation-negative test result (29% in our series), can be reassured of a low vulvar cancer risk after primary HSIL diagnosis. As there are no alternative objective tests for cancer risk stratification of VIN, this is an important result. Clinical validation is currently ongoing in the VENUS study.

Methylation could also guide treatment decisions of patients with multicentric HSIL (intraepithelial lesions at other anogenital sites, such as the cervix (CIN), vagina (VaIN), and/or anus (AIN)), affecting 25 to 66% of VIN patients.(90, 91) A positive methylation test would imply adequate treatment and close follow-up of concurrent anogenital HSIL, while a negative methylation result would point towards conservative management, including watchful waiting. In a small study including 12 patients with multifocal HSIL (multiple vulvar HSIL) we observed limited heterogeneity in methylation profiles between HSIL cases (**Chapter 5**).

(81) Exploring heterogeneity in multifocal VIN using biomarkers warrants further prospective studies with larger sample sizes and long-term follow-up. Multicentric HSIL is currently being investigated in the VENUS cohort.

HPV-independent VIN

In HPV-independent VIN, cases with mutant p53 staining displayed an almost 8-fold higher 5-year cancer risk compared to cases with p53 wild-type staining (**Chapter 8)**.(81) A recent study of our research group on 114 HPV-VIN patients confirmed the higher cancer risk in p53 mutant HPV-independent VIN as compared to p53 wild-type HPV-independent VIN.(20) This study also displayed a 2.2-fold increased risk of developing recurrent HPV-independent VIN when p53 was mutant, even though the results were not significant because of small group numbers.(20) The historical cohort also demonstrates this result, although analyses on recurrent VIN are still ongoing.

P53 IHC is a simple and cheap test and a powerful biomarker for accurate diagnosis, contributing to early detection of aggressive p53 mutant HPV-independent lesions, including predisposing lesions with elevated cancer risk, such as a subset of LS lesions. It was shown that vulvar cancer-associated LS more frequently exhibited mutant p53 IHC compared to LS without vulvar cancer (32% versus 3%, respectively, p=0.002).(77) Although it is too early to draw firm conclusions, it is expected that p53 IHC will alter the management of both patients with HPV-independent VIN and HPV-independent vulvar cancer. For now, accurate surgical excision and close follow-up seem warranted for both p53 mutant and p53 wild-type HPV-independent VIN.

As outlined in **Chapter 8**, in HPV-independent VIN, methylation did not add any value to p53 for predicting cancer.(81) In addition, the median time to cancer did not differ between methylation-negative and -positive HPV-independent VIN. In p53 mutant HPV-independent VIN, methylation-positivity was 90%, which is a reflection of the very high short-term cancer risk of p53 mutant HPV-independent VIN. In p53 wild-type HPV-independent VIN, methylation-positivity was 75%. In this patient group, none of the four methylation-negative cases progressed to cancer within 10 years, whereas four of twelve (33%) methylation-positive cases did show progression to cancer within 5 years (p=0.18). Results were not statistically significant because of small sample sizes, however, the diagnostic and prognostic value of p53 will be further examined in the VENUS.(81)

Future perspectives

Encouragingly, more research is being conducted into the etiology and molecular background of HSIL and HPV-independent VIN. Nevertheless, much is still unknown, and in order to make the shift towards precision oncology, to improve clinical outcome of patients, optimization of VIN characterization is needed. Implementation of the three-tiered classification in international classification systems and guidelines is necessary for that purpose.

To aim for the prevention of vulvar cancer, we should focus on a high worldwide HPV vaccination rate, as vaccination results are promising.(92-94) In addition, we should better identify high-risk predisposing lesions. Unpublished data show that 70% of patients presenting with vulvar carcinoma did not have a prior biopsy, making more accurate and earlier detection of the aggressive HPV-independent VIN of utmost importance. Crucial for early detection of HPV-independent lesions is to gain more insight into the symptoms that precede cancer, providing proper education on vulvar dermatoses, and increasing overall awareness. Moreover, we need to identify LS patients with increased cancer risk. Methylation can serve as a prognostic marker in LS, which is shown by multiple reports.(20, 75, 95, 96) A recently published study of our research group, using our three-gene methylation marker panel, observed that 75% of LS lesions with concurrent vulvar cancer, or with vulvar cancer during follow-up, were methylation-positive, compared to 17% of LS lesions without cancer ($p<0.001$).(77) To improve identification of high-risk LS patients, a proof-of-concept study, the 'VULVA-SCREEN study', has recently been initiated to explore the use of methylation testing in vulvar scrapes and urine. Although the use of vulvar cytology is not recommended to date, (97), preliminary analyses (not published) are promising, as detection of vulvar (pre-)cancer in vulvar scrapes and urine by DNA methylation testing seems feasible.

Although many methylation markers for anogenital (pre-)cancer have been discovered to date, only a few are currently commercially available. An example of a methylation test that is currently used in the clinic is the Precursor-M+/QIAsure Methylation Test®, intended for cervical scrapes to guide treatment decisions in selected patients. Our future goal is to develop similar methylation tests to risk stratify vulvar and anal lesions. For research purposes, such a test is the currently commercially available PreCursor-M AnoGYN (Research Use Only) test, measuring methylation markers *ASCL1* and *ZNF582*.(98) Clinical validation for risk stratification of vulvar and anal lesions is currently ongoing in the VENUS and MARINE studies, respectively.(99) To ultimately obtain CE-IVD certification for these new methylation marker tests, to reach the clinic, it is important to consider the regulatory requirements early in the research process.

In summary, the studies presented in this thesis underscore the importance of biomarker-based testing of high-grade VIN. Moreover, the studies constitute a foundation for further research. As we continue to refine and validate the biomarkers, we will progress towards effective risk stratification of HSIL and HPV-independent VIN. To date (August 2024), the VENUS has enrolled as many as 235 patients from 15 centers, making it the largest prospective series in VIN worldwide. The VENUS will enable validation of the biomarkers, which is needed for successful implementation of methylation-based testing into clinical practice in the near future.

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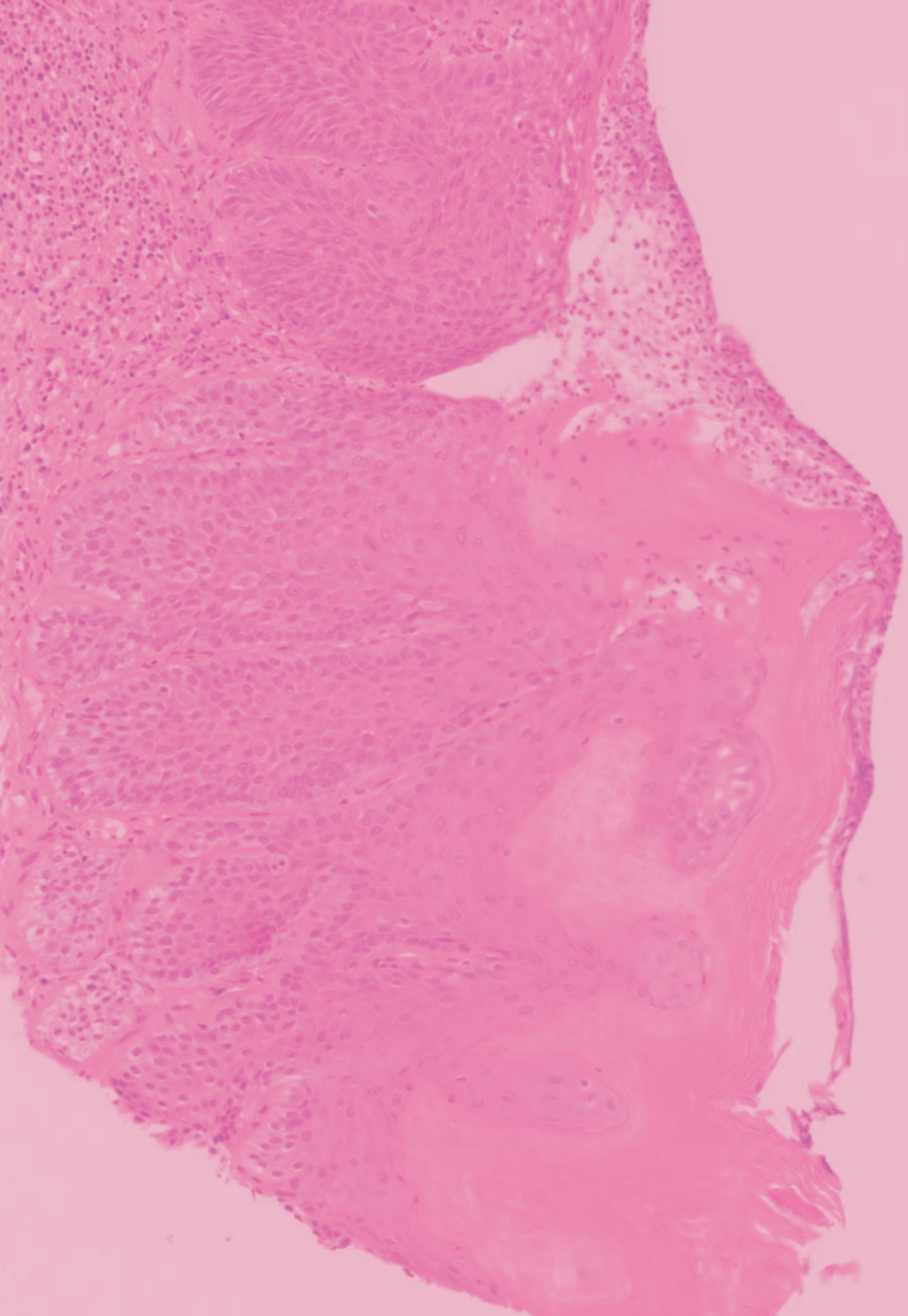


CHAPTER 11

English Summary

High-grade vulvar intraepithelial neoplasia (VIN) is the precursor of vulvar cancer and is divided into human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesion (HSIL) and HPV-independent VIN. HPV-independent VIN is often referred to as differentiated VIN (dVIN) and arises independent from HPV infection, mostly on a background of lichen sclerosus (LS). High-grade VIN is a heterogeneous disease with a varying risk of progression to cancer, shows frequent recurrences upon treatment, and is accompanied by significant psychosocial distress and decreased quality of life. To date, there are no prognostic tests stratifying patients into low or high vulvar cancer risk. Instead, all patients are treated similarly, with overtreatment as a result, leading to morbidity and reduced sexual function. Hence, there is an urgent clinical need for objective biomarkers for cancer risk stratification in patients with high-grade VIN.

In **Chapter 2**, the incidence of high-grade VIN was calculated from a longitudinal, population-based historical cohort series including 1,148 patients with an original diagnosis of high-grade VIN between 1991 and 2011. Vulvar cancer risk and associated risk factors were studied in the patients with high-grade VIN without previous or concurrent vulvar cancer (n=894). During the study period, the incidence of both HSIL and HPV-independent VIN had increased. The 10-year vulvar cancer risk was 10% for HSIL and 50% for HPV-independent VIN. Independent risk factors for progression to vulvar cancer were type of VIN, age and presence of lichen sclerosus.

In **Chapter 3** we performed a systematic literature search reviewing the primary and recurrent vulvar cancer risk in patients with HPV-independent VIN, given the limited available evidence on this topic. A systematic search starting with 455 relevant papers resulted in seven eligible studies. Reported vulvar cancer risks in HPV-independent VIN varied between 33 and 86%, with a median time to progression to vulvar cancer of 9 to 23 months. The risk of developing recurrent vulvar cancer in HPV-independent VIN (with associated vulvar cancer) was 32–94%. This systematic review confirmed the high risk of patients with HPV-independent VIN to develop vulvar cancer, including the short time to progression to cancer, as was also demonstrated in Chapter 2.

In **Chapter 4**, twelve candidate DNA methylation markers (*ASCL1, CADM1, FAM19A4, GHSR, LHX8, MAL, miR124-2, PHACTR3, PRDM14, SST, ZIC1* and *ZNF582)* associated with HPV-induced anogenital carcinogenesis were tested for high-grade VIN and vulvar cancer detection with quantitative multiplex methylation-specific PCR (qMSP) in a cross-sectional series. This series included 192 vulvar samples: 58 vulvar cancers, 30 VIN adjacent to vulvar cancer, 41 VIN without associated vulvar cancer (37 HSIL

and 4 HPV-independent VIN) and 63 healthy vulvar tissues. Methylation markers showed significantly higher methylation levels with increasing severity of disease, both in HPV-associated and HPV-independent lesions. VIN without associated vulvar cancer showed heterogeneous methylation levels, while VIN adjacent to vulvar cancer showed similar high methylation levels as vulvar cancer.

An exploratory study on 12 patients with multifocal vulvar HSIL was described in **Chapter 5**. Six methylation markers (*ASCL1, LHX8, ZNF582, GHSR, SST and ZIC1*), HPV genotype and expression of immunohistochemical markers $p16^{INK4a}$ and Ki-67 were examined in 27 individual HSILs. All except one patient showed similar methylation levels in the individual lesions, indicating little variation within patients. On the other hand, methylation levels were markedly different between patients. Most patients (10 out of 12) harbored the same HPV genotype in the individual lesions. The presence of different HPV genotypes in individual HSILs in 2 patients indicates that these lesions developed independently. All HSIL demonstrated diffuse $p16^{INK4a}$ staining and increased Ki-67 staining. The outcomes of this study showed that the biomarkers varied between patients, but were comparable within most patients.

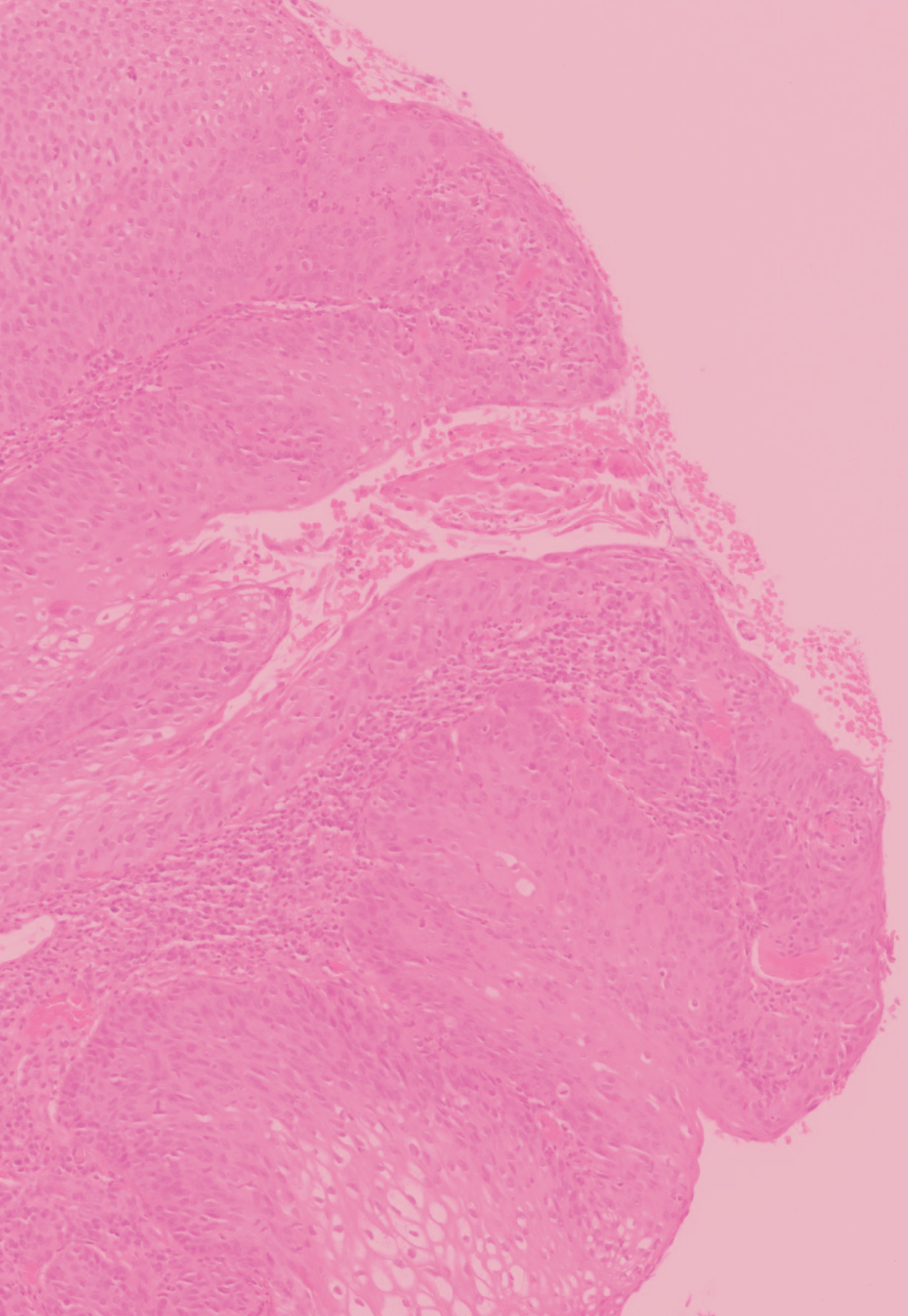
In the research described in **Chapter 6**, tissue blocks from 751 of the 894 original high-grade VIN without associated vulvar cancer from the historical cohort described in Chapter 2, were retrieved. The vulvar lesions were categorized by histopathological reassessment, integrating results of immunohistochemistry of $p16^{INK4a}$, p53, and Ki-67, and HPV DNA testing. In addition, vulvar cancer risks were calculated. Integrated analyses resulted in 88% HPV-associated lesions, 11% HPV-independent lesions, and 1% inconclusive lesions. HSIL nearly always demonstrated $p16^{INK4a}$ block-positivity, HPV-positivity, p53 wild-type staining, often with a mid-epithelial staining pattern, and increased Ki-67 staining extending into the upper half of the epithelium. HPV-independent VIN had mutant p53 staining in 65%, never demonstrated p53 mid-epithelial staining, and showed a wide morphological spectrum, ranging from differentiated to non-differentiated ('HPV-associated-like', in 41%). Immunohistochemical markers and HPV genotyping were useful for a correct diagnosis, but also showed pitfalls one needs to be aware of. The 10-year cancer risk was 8%, 67% and 28% in patients with HSIL, p53 mutant HPV-independent VIN, and p53 wild-type HPV-independent VIN, respectively, and was highest (73%) in HPV-independent VIN with non-differentiated ('HPV-associated-like') morphology. The results of this study showed that optimal categorization into HPV-associated and HPV-independent VIN is of utmost importance given the different cancer risks and the frequent similar histomorphology. Moreover, $p16^{INK4a}$, p53, Ki-67 and HPV can guide a correct diagnosis.

The outcomes of Chapter 4 revealed the value of methylation analysis for the detection of high-grade VIN and vulvar cancer. In **Chapter 7,** the 12 DNA methylation markers were validated in a cross-sectional analysis on the 751 vulvar tissue samples of the historical cohort (Chapter 6), together with 113 healthy vulvar controls. *SST* was the best-performing individual marker with an area under the curve (AUC) of 0.90, detecting only 2% of controls and 80% of high-grade VIN, including 95% of HPV-independent VIN. Selection of a marker panel, including *ZNF582, SST* and *miR124-2*, resulted in a comparably high accuracy for the detection of high-grade VIN (AUC 0.89). These findings demonstrated that DNA methylation is an objective biomarker which can distinguish reactive or low-grade lesions not in need of treatment, from high-grade lesions. Furthermore, this work encouraged further prognostic validation of methylation biomarkers for cancer risk stratification of patients with VIN.

In **Chapter 8**, the three-gene methylation marker panel (*ZNF582*, *SST*, and *miR124-2*), as determined in Chapter 7, and other risk factors (age, p53 immunohistochemistry status, HPV genotype and presence of lichen sclerosus) in relation to cancer risk, were evaluated by Kaplan-Meier and Cox regressio in all 578 HSIL and 46 HPV-independent VIN from the historical cohort. In patients with HSIL, a positive methylation status harboured a 4.87 times higher vulvar cancer risk after five years compared to HSIL with a negative methylation status. The prognostic value of methylation remained present when selecting patients who did not receive radical surgical excision as their primary treatment. Patients with methylation-negative HSIL carried a low cancer risk and can be safely treated with a non-radical treatment modality, which could reduce morbidity and increase quality of life. In HPV-independent VIN patients, p53 status was the sole prognostic risk factor (HR 7.67) for progression to cancer.

In **Chapter 9,** the performance of immunohistochemical markers CK17 and SOX2 was validated in a series of 150 vulvar lesions from the historical cohort, including all 46 HPV-independent VIN, 37 non-dysplastic lesions, 6 inconclusive lesions, and a subset of all HPV-associated lesions, i.e. 58 HSIL and 4 LSIL. These 150 cases were reviewed by a panel of six gynecological pathologists. The accuracy for each individual marker and for a combination of markers was calculated for the diagnosis of HPVi VIN with non-dysplastic cases as controls. Significantly more CK17- and SOX2-positive cases were observed in HPVi VIN compared to non-dysplastic cases (respectively 83% and 33% versus 25% and 3%; $p<0.001$). Highest diagnostic accuracy (89%) for HPVi VIN was obtained when combining p53 and CK17 IHC markers.

In **Chapter 10**, the main findings of this thesis are discussed and related to current and future perspectives.



Appendices

Nederlandse samenvatting | Dutch summary

Hooggradige vulvaire intra-epitheliale neoplasie (VIN) is de voorloperlaesie van vulvakanker. VIN wordt onderverdeeld in humaan papillomavirus (HPV)-geassocieerde hooggradige squameuze intra-epitheliale laesie (HSIL) en HPV-onafhankelijke VIN. HSIL wordt veroorzaakt door een hoog-risico HPV infectie die niet geklaard kan worden door het lichaam. HPV-onafhankelijke VIN ontstaat meestal op een achtergrond van lichen sclerosus, onafhankelijk van een HPV-infectie. HPV-onafhankelijke VIN wordt vaak nog aangeduid als gedifferentieerde VIN (dVIN), zoals het voorheen heette. Hooggradige VIN is een heterogene ziekte met een hoog recidief risico en een variabel risico op progressie naar vulvakanker. De ziekte gaat gepaard met aanzienlijke psychosociale stress en vaak een verminderde kwaliteit van leven. Tot op heden zijn er geen prognostische testen beschikbaar die de kans op vulvakanker kunnen bepalen. Het gevolg is dat patiënten op een vergelijkbare manier behandeld worden, wat kan leiden tot overbehandeling en morbiditeit, waarbij te denken valt aan een verminderde seksuele functie. Concluderend is er een dringende klinische behoefte aan objectieve biomarkers voor risicostratificatie van patiënten met hooggradige VIN.

In **Hoofdstuk 2** werd de incidentie van hooggradige VIN berekend aan de hand van een longitudinale, populatie-brede, historische cohortreeks, bestaande uit 1.148 patiënten met een originele diagnose hooggradige VIN tussen 1991 en 2011. Het risico op vulvakanker en risicofactoren voor het ontwikkelen van vulvakanker werden onderzocht bij patiënten met hooggradige VIN, die zowel geen gelijktijdige vulvakanker hadden als geen voorgeschiedenis van vulvakanker (n=894). Tijdens de onderzoeksperiode was de incidentie van HSIL en HPV-onafhankelijke VIN gestegen. Het 10-jaars risico op vulvakanker was 10% voor HSIL en 50% voor HPV-onafhankelijke VIN. Onafhankelijke risicofactoren voor progressie naar vulvakanker waren type VIN, leeftijd boven de 50 jaar en de aanwezigheid van lichen sclerosus.

In **Hoofdstuk 3** hebben we een systematisch literatuuronderzoek verricht naar het risico op primair- en recidief vulvakanker bij patiënten met HPV-onafhankelijke VIN. Een systematische zoekopdracht leverde 455 artikelen op, waarvan slechts 7 artikelen geschikt waren voor deze literatuurstudie. De gerapporteerde risico's op vulvakanker bij HPV-onafhankelijke VIN varieerden tussen 33 en 86%, met een mediane tijd tot progressie naar vulvakanker van 9 tot 23 maanden. Het risico vulvakanker te ontwikkelen in patiënten met HPV-onafhankelijke VIN met een voorgeschiedenis van vulvakanker was 32–94%. Deze systematische review bevestigde dat patiënten met

HPV-onafhankelijke VIN een hoog risico hebben op vulvakanker kort na de initiële diagnose VIN, hetgeen ook al werd aangetoond in Hoofdstuk 2.

In **Hoofdstuk 4** werden twaalf kandidaat DNA methyleringsmarkers (*ASCL1*, *CADM1*, *FAM19A4*, *GHSR*, *LHX8*, *MAL*, *miR124-2*, *PHACTR3*, *PRDM14*, *SST*, *ZIC1* en *ZNF582*), welke reeds bekend waren van onderzoek naar HPV-geassocieerde cervicale en anale laesies, getest op de detectie van hooggradige VIN en vulvakanker middels kwantitatieve multiplex methylatie-specifieke PCR (qMSP). Er werden 192 vulvaweefsels getest: 58 vulvakankers, 30 VIN gelegen naast vulvakanker, 41 VIN zonder vulvakanker (37 HSIL en 4 HPV-onafhankelijke VIN) en 63 gezonde vulvaweefsels. Methyleringsmarkers toonden significant hogere niveaus bij ernstiger ziekte, zowel in HPV-geassocieerde als HPV-onafhankelijke laesies. VIN aanliggend aan kanker en vulvakanker hadden vergelijkbaar hoge methyleringsniveaus, terwijl VIN zonder vulvakanker heterogene methyleringsniveaus had. Deze bevindingen rechtvaardigden verder onderzoek naar DNA-methylatietesten voor het onderscheid tussen VIN met een laag of hoog risico op progressie naar vulvakanker.

Een pilotstudie betreffende 12 patiënten met multifocale vulvaire HSIL werd beschreven in **Hoofdstuk 5**. Zes methyleringsmarkers (*ASCL1*, *LHX8*, *ZNF582*, *GHSR*, *SST* en *ZIC1*), HPV-genotype en expressie van immunohistochemische markers $p16^{INK4a}$ en Ki-67 werden onderzocht in 27 individuele HSIL. Op één na vertoonden alle patiënten vergelijkbare methyleringsniveaus in hun individuele laesies, wat erop wijst dat er weinig variatie in methyleringsniveau was binnen patiënten. Tussen patiënten waren methyleringsniveaus echter aanzienlijk verschillend. De meeste patiënten (10 van de 12) hadden hetzelfde HPV-genotype in hun individuele laesies. De aanwezigheid van verschillende HPV-genotypen in individuele HSIL bij twee patiënten geeft aan dat deze laesies zich onafhankelijk van elkaar ontwikkeld hebben. Alle HSIL vertoonden diffuse $p16^{INK4a}$- en verhoogde Ki-67 expressie. De uitkomsten van dit onderzoek lieten heterogeniteit van methyleringsniveaus in multifocale HSIL tussen patiënten zien, terwijl de biomarkers binnen de meeste patiënten een vergelijkbare expressie hadden.

In het onderzoek beschreven in **Hoofdstuk 6** werden weefselblokjes verzameld van 751 van de 894 (84%) originele hooggradige VIN zonder vulvakanker uit het historisch cohort, dat reeds beschreven is in Hoofdstuk 2. De vulvalaesies werden gecategoriseerd door middel van histopathologische herbeoordeling, met integratie van de resultaten van immunohistochemische kleuringen $p16^{INK4a}$, p53 en Ki-67 en HPV DNA-testen. Ook werden per categorie de risico's op vulvakanker berekend. Geïntegreerde analyses resulteerden in 88% HPV-geassocieerde laesies, 11% HPV-

onafhankelijke laesies en 1% niet-classificeerbare laesies. HSIL vertoonde bijna altijd blok-positiviteit voor $p16^{INK4a}$, een positieve HPV test uitslag, wildtype p53 expressie, vaak met een zogenaamd 'mid-epitheliaal' aankleuringspatroon, en verhoogde Ki-67 expressie tot in de bovenste helft van het epitheel. HPV-onafhankelijke VIN had een p53 mutant aankleuringspatroon in 65%, had nooit p53 mid-epitheliale aankleuring en vertoonde morfologisch een breed spectrum, variërend van gedifferentieerd tot niet-gedifferentieerd ('HPV-geassocieerd-achtig', in 41%). Immunohistochemische markers en HPV-genotypering waren nuttig voor een correcte diagnose, maar brachten ook valkuilen aan het licht waar men zich van bewust moet zijn. Het 10-jaars kankerrisico was respectievelijk 8%, 67% en 28% bij patiënten met HSIL, p53 mutante HPV-onafhankelijke VIN en p53 wild-type HPV-onafhankelijke VIN, en was het hoogst (73%) bij HPV-onafhankelijke VIN met niet-gedifferentieerde ('HPV-geassocieerd-achtige') morfologie. De resultaten van deze studie toonden aan dat een optimale indeling in HPV-geassocieerde en HPV-onafhankelijke VIN van groot belang is gezien de verschillende kankerrisico's en de vaak vergelijkbare histomorfologie. Toepassing van $p16^{INK4a}$, p53, Ki-67 en HPV kunnen behulpzaam zijn bij stellen van de juiste diagnose.

De bevindingen in Hoofdstuk 4 toonden de waarde van methyleringsanalyse van 12 genen voor de detectie van hooggradige VIN en vulvakanker. In **Hoofdstuk 7** werden deze 12 DNA-methyleringsmarkers gevalideerd in de serie van 751 vulvaire laesies uit het historische cohort (Hoofdstuk 6), samen met 113 gezonde vulvaweefsels. *SST* was de best presterende individuele methyleringsmarker met een area under the curve (AUC) van 0,90, waarbij slechts 2% van de controles en 80% van de hooggradige VIN, inclusief 95% van de HPV-onafhankelijke VIN, werden gedetecteerd. Een panel van markers, waaronder *ZNF582*, *SST* en *miR124-2*, toonde een vergelijkbaar hoge nauwkeurigheid als marker *SST* voor de detectie van hooggradige VIN (AUC 0,89). Deze resultaten toonden aan dat DNA methylering een objectieve biomarker is die reactieve of laaggradige laesies, die geen behandeling vereisen, kan onderscheiden van hooggradige laesies. Dit werk ondersteunde verder onderzoek, inclusief prognostische validatie van methyleringsmarkers voor risicostratificatie van patiënten met VIN ten aanzien van het risico op progressie naar vulvakanker.

In **Hoofdstuk 8** werd het drie-genen methylering markerpanel (*ZNF582*, *SST* en *miR124-2*) uit Hoofdstuk 7, samen met andere risicofactoren (leeftijd, p53 immuunhistochemie status, HPV-genotype en de aanwezigheid van lichen sclerosus), geanalyseerd in relatie tot het kankerrisico met behulp van Kaplan-Meier en Cox regressieanalyses bij 578 HSIL en 46 HPV-onafhankelijke VIN uit het historische cohort. Bij patiënten met HSIL was een positieve methyleringsstatus geassocieerd

met een 4,87 keer hoger risico op vulvakanker na vijf jaar in vergelijking met HSIL met een negatieve methyleringsstatus. De prognostische waarde van methylering bleef aanwezig in de patiënten die geen radicale chirurgische excisie als primaire behandeling hadden ondergaan. Patiënten met methyleringsnegatieve HSIL hadden een laag kankerrisico, en konden derhalve veilig worden behandeld met een niet-radicale behandelingsmethode, wat morbiditeit zou kunnen verminderen en de kwaliteit van leven zou kunnen verbeteren. Bij HPV-onafhankelijke VIN was p53 status de enige prognostische risicofactor voor progressie naar kanker (hazard ratio 7,67).

In **Hoofdstuk 9** werden immunohistochemische markers CK17 en SOX2 gevalideerd in een reeks van 150 vulvaire laesies uit het historische cohort, inclusief alle 46 HPV-onafhankelijke VIN, 37 niet-dysplastische laesies, 6 niet-classificeerbare laesies, en een subset van alle HPV-geassocieerde laesies, namelijk 58 HSIL en 4 LSIL. Deze 150 gevallen werden beoordeeld door een panel van zes gynaecopathologen. De accuracy (nauwkeurigheid) van elke individuele marker en van een combinatie van markers werd berekend voor de diagnose HPV-onafhankelijke VIN, met niet-dysplastische gevallen als controles. Er waren significant meer CK17- en SOX2-positieve HPV-onafhankelijke VIN vergeleken met niet-dysplastische laesies (respectievelijk 83% en 33% versus 25% en 3%; $p<0,001$). De hoogste diagnostische nauwkeurigheid (89%) voor HPV-onafhankelijke VIN had de combinatie van immunohistochemische markers p53 en CK17.

In **Hoofdstuk 10** worden de belangrijkste bevindingen van dit proefschrift nader belicht en afgezet tegen de huidige literatuur. Mogelijke klinische implicaties en richtingen voor vervolgonderzoek zullen worden besproken.

List of publications

Thuijs, N.B., Uitdehaag, B.M.J., van Ouwerkerk, W.J.R., van der Valk, P., Vandertop, W.P., Peerdeman, S.M. (2012) Pediatric meningiomas in The Netherlands 1974–2010: a descriptive epidemiological case study. Childs Nervous System. 28(7): 1009-1015.

Bouman, A., Alders, M., Oostra, R.J., van Leeuwen, E., **Thuijs, N.B.**, van der Kevie-Kersemaekers, A.M., van Maarle, M. (2017) Oral-facial-digital syndrome type 1 in males: Congenital heart defects are included in its phenotypic spectrum. American Journal of Medical Genetics. Part A. 173(5):1383-1389.

Machiels, M., van Montfoort, M.L., **Thuijs, N.B.**, van Berge Henegouwen, M.I., Alderliesten, T., Meijer, S.L., van Hooft, J.E., Hulshof, M.C.C.M. (2019) Microscopic tumor spread beyond (echo)endoscopically determined tumor borders in esophageal cancer. Radiation Oncology. 4;14(1):219.

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Thuijs, N.B., Berkhof, H., Özer, M., Duin, S., van Splunter, A.P., Snoek, B.C., Heideman, D.A.M., van Beurden, M., Steenbergen, R.D.M., Bleeker, M.C.G. (2021) DNA methylation markers for cancer risk prediction of vulvar intraepithelial neoplasia. International Journal of Cancer. 10;148(10):2481-2488.

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*shared first author

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Fons, G., **Thuijs, N.B.**, Tjiong, M., Stalpers, L.J.A., van der Velden, J. (2023) Selective Removal of Only Clinically Suspicious Positive Lymph Nodes Instead of a Complete Inguino-Femoral Lymph Node Dissection in Squamous Cell Carcinoma of the Vulva. Cancers (Basel). 28;15(15):3844.

Thuijs, N.B., van Beurden, M., Duin, S., Heideman, D.A.M., Berkhof, H., Steenbergen, R.D.M., Bleeker, M.C.G. (2023) High-grade vulvar intraepithelial neoplasia: comprehensive characterization and long-term vulvar carcinoma risk. Histopathology. 84(2):301-314.

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*shared first author

Runello, F., Jary, A., Duin, S., Kim, Y., Voss, F.O., **Thuijs, N.B.**, Bleeker, M.C.G., Steenbergen, R.D.M. (2025) DNA Methylation and Copy Number Alterations in the progression of HPV-associated Vulvar Intraepithelial Neoplasia. *International Journal of Cancer. Epub ahead of print*.

Thuijs, N.B.* & Voss, F.O.*, Ewing-Graham, P.C., Dasgupta, S., Berkhof, H., Bulten, H., van de Vijver, K., Bleeker, M.C.G. (2024) Expression of CK17 and SOX2 in vulvar intraepithelial neoplasia: a comprehensive analysis of 150 vulvar lesions. *Cancers (Basel). 2024 Nov 26;16(23):3966.*
*shared first author

PhD Portfolio

Name PhD student:	Nikki Bo Thuijs
PhD period:	October 2017 – August 2023
PhD supervisors:	Dr. Maaike C.G. Bleeker
	Prof.dr. Renske D.M. Steenbergen
	Dr. Marc van Beurden
	Prof.dr. Johannes Berkhof

COURSES AND WORKSHOPS

2018	Research Integrity Course
2018	Writing a Scientific Article, Taalcentrum VU, Amsterdam
2018	The art of presenting science, Oncology Graduate School, Amsterdam
2019	Basic Medical Statistics Course, NKI, Amsterdam
2020	Cursus Klinische Predictiemodellen, EpidM
2022	GraphPad Prism, ASAP, Amsterdam UMC, location VUmc, Amsterdam

CONGRESSES

2018	Eurogin, Lisbon, Portugal
	Oral presentation: *DNA methylation markers for risk stratification of vulvar intraepithelial neoplasia*
2019	European Society of Gynecological Oncology (ESGO)
	Poster: *Vulvar intraepithelial neoplasia: incidence and long-term risk of vulvar squamous cell carcinoma*
	Poster: *Host cell DNA methylation markers for cancer risk stratification of vulvar intraepithelial neoplasia*
2021	International International Papillomavirus Conference (IPVC)
	Poster: *Comprehensive analysis of 753 cases of high-grade vulvar intraepithelial neoplasia, including p16^{INK4a} and human papillomavirus status*
2023	Eurogin, Bilbao, Spain
	Oral presentation: *Comprehensive characterization of 751 vulvar lesions, originally diagnosed as high-grade vulvar intra-epithelial neoplasia*

OTHER SCIENTIFIC MEETINGS

2018 Science Exchange Day, Amsterdam UMC, location VUmc, Amsterdam
Poster: *DNA methylation markers for risk stratification of vulvar intraepithelial neoplasia*

2018 Oncology Graduate School Retreat, Renesse
Poster: *Vulvar intraepithelial neoplasia: Incidence and long term risk of vulvar squamous cell carcinoma*

2018 Cancer Center Amsterdam Retreat, Noordwijkerhout
Poster: *DNA methylation markers for risk stratification of vulvar intraepithelial neoplasia*

2018 Najaarssymposium Nederlandse Vereniging voor Vulvapathologie
Presentation: *Methylering in VIN*

2020 Pathology Research Meeting 2020
Presentation: *Cancer risk prediction in vulvar intraepithelial neoplasia (VIN)*

2022 Pathology Research Meeting 2022 Presentation: *Biomarkers in vulvar intraepithelial neoplasia; towards early identification of patients at risk of vulvar cancer*

2023 Najaarssymposium Nederlandse Vereniging voor Vulvapathologie
Presentation: *High-grade vulvar intraepithelial neoplasia: comprehensive characterization and long-term vulvar carcinoma risk*

2023 CCA Seminar Series Call
Presentation: *DNA methylation is a strong prognostic factor for progression to vulvar cancer in patients with high-grade vulvar intraepithelial neoplasia*

SUPERVISION OF STUDENTS

2018 Bachelor thesis medical student: *The effect of exercising on memory in the young and healthy population*

2018 Tutor of second year medical students (6 months)

2020 Scientific internship medical student (4 months)

2021 Scientific internship medical student (4 months)

PRIZES

2018	Jonge Onderzoekersprijs (€1.000) Nederlandse Vereniging voor Vulvapathologie
Abstract:	*DNA methylation markers for risk stratification of vulvar intraepithelial neoplasia*

OTHER

2018-2023	Start and coordinating the VENUS (Vulvar intraEpithelial Neoplasia in sitU Study), a national, multicenter, prospective study, including patients with HSIL and HPV-independent VIN
2018-2023	Pizza and Ice Cream meetings
2018-2023	Weekly meeting with HPV research group
2018-2023	Weekly CCA Pathology meetings
2018-2023	Two-weekly laboratory meetings with HPV research group
2018-2023	Monthly journal club HPV research group
2019-2023	VIN Working group for Richtlijnendatabase (Oncoline)
2021-2023	Peer-reviewer for scientific journals

Curriculum vitae / About the author

Nikki Bo Thuijs was born on February 12th, 1987 in Hilversum. She grew up in Maartensdijk with her parents, sister and brother. Nikki finished secondary school at Het Nieuwe Lyceum in Bilthoven and was admitted to study medicine at the Vrije Universiteit in Amsterdam in 2005. During medical school Nikki was an active board member of the Annual Representation Committee and the Student Council. She completed her scientific internship at the 'Neurodynamics Research Laboratory' in the Children's Hospital/Harvard Medical School, Boston, United States. Nikki obtained her Medical Doctor degree in 2011. After three years of neurosurgical training at the Neurosurgical Center Amsterdam in Amsterdam UMC, she decided to change course and started her training as a pathologist in 2015 in Amsterdam UMC. During her residency program she was also trained in Tergooi ziekenhuis, OLVG Lab B.V. and Spaarne Gasthuis. Nikki started her PhD trajectory in 2017 at Amsterdam UMC, location VUmc, and Cancer Center Amsterdam, and alternated this trajectory with her training in pathology. Nikki will comple her pathology training in March 2025, after which she will start a fellowship in dermato/gynaecopathology at Amsterdam UMC. She lives in Naarden together with Thomas van Egmond and their three boys, Philip, Julius and Alexander.

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Promotiecommissie

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Vulvar intraEpithelial Neoplasia in sitU Study (VENUS)

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Amsterdam UMC, locatie VUmc - onderzoek

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*

Amsterdam UMC - afdeling Pathologie

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Afdeling Pathologie, overig

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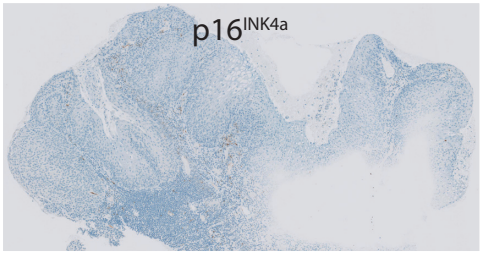
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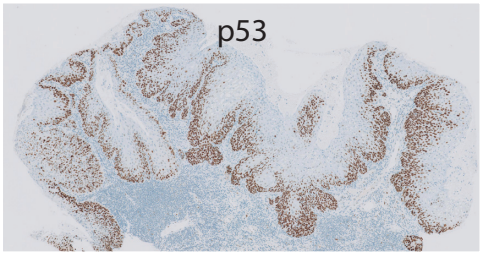
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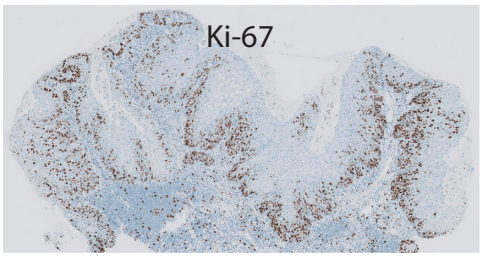
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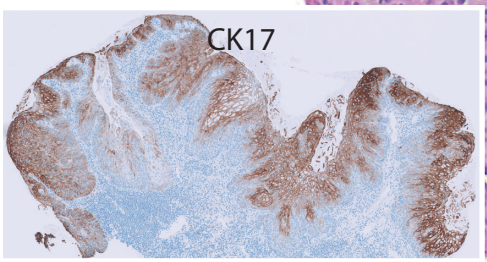
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