Vulvar cancer; pathogenesis, molecular genetics and treatment

Linda Suzanne Nooij

Vulvar cancer;
pathogenesis, molecular genetics and treatment

Linda Suzanne Nooij

Cover Stefanie van den Herik | www.svdhmedia.nl
Layout Paula Baggen en Bregje Jaspers, www.proefschriftontwerp.nl
Printed by ProefschriftMaken, www.proefschriftmaken.nl
ISBN 978-94-6295-921-7

Vulvar cancer; pathogenesis, molecular genetics and treatment
The research described in this thesis was performed at the department of Gynaecology and Pathology at the Leiden University Medical Center, Leiden, the Netherlands.

Financial support for printing of this thesis was kindly provided by Leiden University, the departments of gynaecology and pathology of the Leiden University Medical Center, Raad van Bestuur Haaglanden Medisch Centrum, Nederlandse Vereniging voor Vulvapathologie, Stichting Olijf, Pfizer, Chipsoft.

Vulvar cancer;
pathogenesis, molecular genetics and treatment

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus Prof. Mr. C.J.J.M. Stolker
volgens besluit van het College voor Promoties
te verdedigen op donderdag 28 juni 2018
klokke 16.15 uur

door

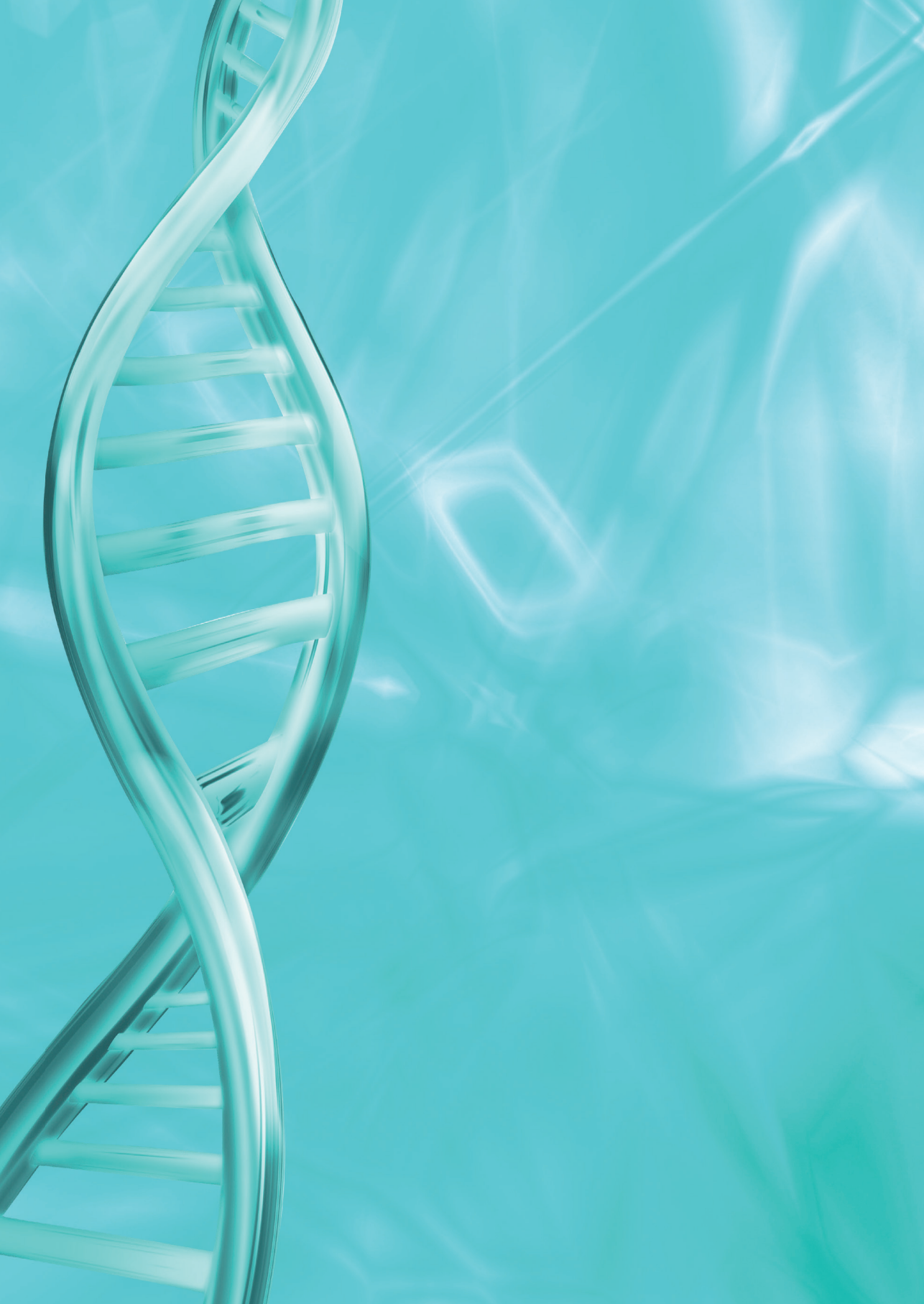
Linda Suzanne Nooij

Geboren te Amsterdam in 1980

Promotor	Prof. Dr. J.B.M.Z. Trimbos
Copromotoren	Dr. M.I.E. van Poelgeest Dr. T. Bosse
Leden promotiecommissie	Prof. Dr. V.T.H.B.M. Smit Prof. Dr. M.J. vd Vijver (VUMC / AMC, Amsterdam) Dr. J.A. de Hullu (Radboudumc, Nijmegen)

Table of contents

Chapter 1	General introduction and outline of the thesis	7
Chapter 2	Tumour-free margins in vulvar squamous cell carcinoma: Does distance really matter?	25
Chapter 3	Groin surgery and risk of recurrence in lymph node positive patients with vulvar squamous cell carcinoma	49
Chapter 4	Risk factors and treatment for recurrent vulvar squamous cell carcinoma	69
Chapter 5	Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: a review of the current literature	101
Chapter 6	Stathmin is a highly sensitive and specific biomarker for vulvar high-grade squamous intraepithelial lesions	139
Chapter 7	Genomic characterization of vulvar (pre)cancers identifies distinct molecular subtypes with prognostic significance	155
Chapter 8	General discussion and summary	181
Appendices	Nederlandse samenvatting	202
	List of abbreviations	208
	List of publications	210
	Dankwoord	212
	Curriculum Vitae	215



CHAPTER 1

General introduction and outline of the thesis

General introduction and outline of the thesis

Vulvar cancer

Vulvar cancer (VC) is a rare gynaecological malignancy that accounts for 3-5% of all female genital tract malignancies (1-3) with an incidence rate of 1-3 per 100,000 women in developed countries. This incidence rises with age, with a peak incidence between 60 and 70 years of age (1, 4-6). In the Netherlands (17 million inhabitants) around 300 new patients are diagnosed with vulvar cancer each year (7). Over the last decades the overall incidence has risen (Figure 1), probably because of a higher life expectancy and due to an increase in human papilloma virus (HPV) infections (4, 5). The majority of VCs (90%) are vulvar squamous cell carcinomas (VSCC)(1, 6). Less frequent histological types are malignant melanoma, Bartholin gland carcinoma, invasive Paget's disease, and basal cell carcinoma. Sarcomas and verrucous carcinomas are extremely rare (1, 6, 8). `

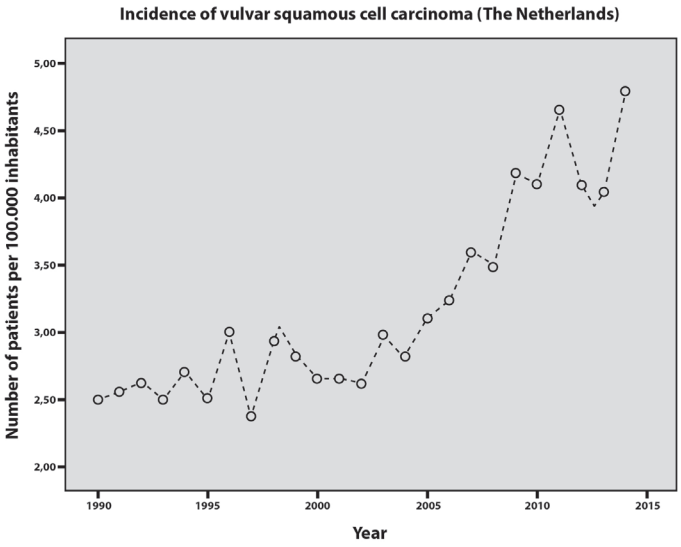


Figure 1: Incidence of vulvar cancer (The Netherlands) (7)

Dissemination of VC occurs through three different routes. The most common pattern of spread is spread by direct extension and lymphogenic to the inguinofemoral lymph nodes. Pelvic lymph node metastases are uncommon, with an incidence of 2-12%, and are seldom found in the absence of groin lymph node metastases (1, 6, 9). Haematogenous spread is very rare, especially in the absence of a groin lymph node metastasis (1, 6, 8-10).

FIGO stage

The International Federation of Gynecology and Obstetrics (FIGO) staging system has been adjusted in 2009 (Table 1) (1, 11, 12). Because prognosis is strongly dependent on the status of the lymph node(s) (13) the number and morphology (size and presence of extra-capsular growth) of involved lymph nodes are taken into account. The FIGO 2009 classification provides an adequate prognostic discrimination between the different stages (12, 14).

Table 1: FIGO 2009 staging system of vulvar cancer

Stage	
I	Tumours confined to the vulva or perineum, no nodal metastasis Ia: Tumour ≤ 2 cm with stromal invasion ≤ 1 mm Ib: Tumour > 2 cm or stromal invasion > 1mm
II	Tumour of any size with extension to adjacent perineal structures (lower urethra, lower vagina, anus), no nodal metastasis
III	Tumour of any size with or without extension to adjacent perineal structures (lower urethra, lower vagina, anus), with inguino-femoral nodal metastasis IIIa: 1 node metastasis (≥ 5 mm) or 1-2 node metastasis(es) (< 5 mm) IIIb: ≥ 2 node metastases (≥ 5 mm) or ≥ 3 node metastases (< 5 mm) IIIc: node metastases with extra-capsular spread
IV	Iva: Tumour invades any of the following: upper urethra and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to pelvic bone, or fixed or ulcerated inguinofemoral nodes IVb: Any distant metastasis including pelvic nodes

Treatment of vulvar cancer

Surgery is the cornerstone of treatment for VC (1, 8). Before 1980, surgery for all VC stages was extensive and consisted of radical vulvectomy with en-bloc lymfadenectomy of the groins and enlarged pelvic nodes (1, 8, 15). The rationale behind this radical surgical procedure was to remove all possible cancer infiltrated tissue by removing the vulvar lesions, the inguinofemoral lymph nodes and the lymphatics in between (8). This treatment strategy led to a high risk of morbidity with reported complication frequencies of up to 90% (16, 17). Most common complications are wound infections, wound breakdown, lymphocysts, lymphedema and psychosexual consequences (6, 8, 15). Furthermore, closure of large skin defects after radical vulvectomy was often insufficient, which could result in postoperative necrosis (6).

During the last decades, treatment for VC has evolved into a more conservative and individualized multidisciplinary approach, without compromising prognosis (1, 6, 8, 9, 15). Nowadays, the extent of disease determines the extend of surgery needed (Figure 2) (9). Micro-invasive VC (stage 1A), defined as a single lesion of ≤ 2 cm with a depth of invasion of ≤1 mm, can be treated with a wide local excision only. Treatment of the groins can be safely omitted, because there is almost no chance of groin metastases in

these patients (1, 6, 15). Surgery for early-stage VC infiltrating > 1 mm consists of wide local excision with uni- or bilateral inguinofemoral lymphadenectomy (IFL) via separate groin incisions or staging of the lymph nodes with the sentinel lymph node (SN) procedure (1, 8). The rationale to justify the use of separate incisions in groin treatment of VC is that the mechanism of lymphatic spread is by embolization rather than by permeation (18). The overall incidence of lymph node metastasis is about 30% (6, 9) and the risk for lymph node metastases rises as the stage of disease, size of the lesion and depth of invasion increases (1, 6, 9). Appropriate groin treatment in order to prevent a groin recurrence is the most important factor in reducing mortality from early stage VC due to the high mortality rate of a groin recurrence. A SN procedure is considered safe in patients with a unifocal vulvar tumour < 4 cm without enlarged or clinically suspicious lymph nodes upon palpation, ultrasonography or CT-scan, with groin recurrence rates of 2,3-3% (16, 19, 20). Unilateral IFL is safe for patients with a lateralized tumour (medial margin of the tumour > 1 cm from the midline) without suspicious groins at physical examination (8, 15). The chance of having positive contralateral lymph nodes for patients with unilateral tumours and negative ipsilateral lymph nodes is low (0.9 – 2.8%) (16, 21, 22). Bilateral IFL should be performed in case of midline tumours, lateral tumours of > 4 cm and in case of positive ipsilateral lymph nodes (1). Due to these treatment adjustments and especially due to the introduction of the SN procedure, morbidity has dramatically decreased. Still, postoperative morbidity remains a major concern, particularly after IFL. One or more complications after an IFL are reported in up to 66% of patients (10, 16, 17).

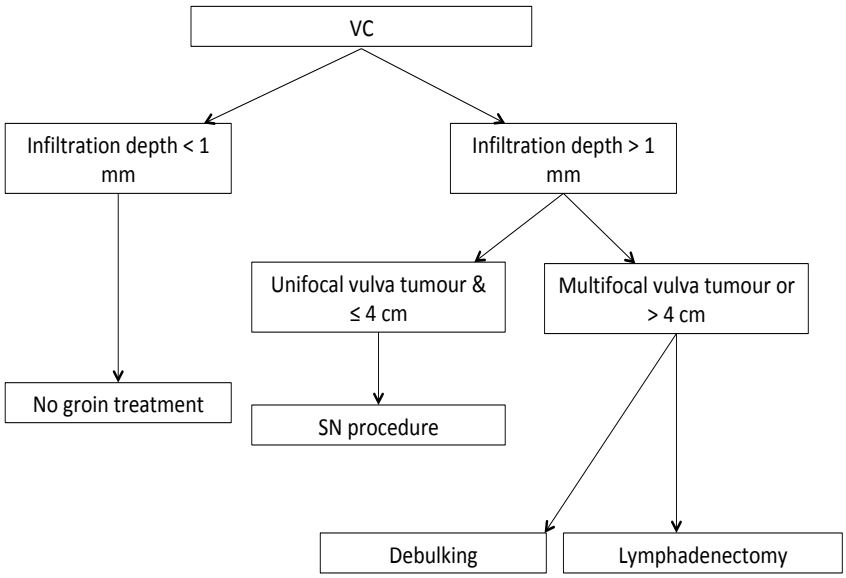


Figure 2: overview of treatment of vulvar cancer (VC). SN: sentinel node

Extensive groin surgery is necessary for patients with a suspicion of groin metastases or in case of a positive SN. Standard treatment at this moment is uni- or bilateral IFL. However, nodal debulking (i.e. removal of enlarged lymph nodes in the groins) might be a good alternative for IFL. A study by Hyde et al. in which nodal debulking was compared with IFL, both followed by radiotherapy, found no difference in groin recurrence rate. However, this study did not evaluate complication rate for both surgical procedures (23).

There are several important clinical issues in the treatment of VC and developing an appropriate, individualized treatment strategy is one of the major challenges. Treatment is often difficult and associated with high complication rates since VC patients are often fragile and elderly patients with high co-morbidity rates (4, 24). This emphasises the need to choose a treatment modality with the lowest morbidity and risk of complications. In the course of the years there have been important developments in less aggressive treatment strategies. Still, there remain major questions on the optimal treatment of VC. Especially the influence of tumour free margins after radical local excision and adequate treatment of the groins are crucial questions. Furthermore, recurrence rate after primary treatment remains high and prevention of these recurrences is a vital clinical challenge in order to further reduce morbidity and complication rates (25, 26).

Adjuvant therapy

Currently, local re-excision is advised in case of positive margins after primary local surgery. Adjuvant radiotherapy can be considered in patients when re-excision is impossible or when re-excision is contra-indicated. Re-excision should also be considered in patients with close tumour-free margins (< 8 mm), especially when there are other risk factors for local recurrence (8, 25, 27-31).

Adjuvant radiotherapy to the groins clearly improves prognosis in patients with involved groin lymph nodes (32, 33) and is indicated after nodal debulking of the groins, in case of two or more groin metastases after inguinofemoral lymphadenectomy or when groin metastases have extranodal growth (1, 8, 9, 15, 27-30).

Prognosis

Prognosis for VC patients is generally good, with an overall five-year survival of 70%. An early diagnosis of VC is important for improved prognosis (6, 9). Five-year survival is 80-90% for patients who present with early-stage VC, regardless of tumour diameter and expansion to the vagina and/or urethra (6, 15, 32, 34). This decreases to 25-67% if groin lymph nodes are affected, largely depending on the number of involved lymph nodes and their growth pattern (6, 9, 12, 32). Five-year survival is 75% for patients with one or two lymph node metastases and decreases to 24% for patients with five or six involved lymph nodes (8). Patients with extranodal growth of a lymph node metastasis

have a 5-year survival of 34% compared to 66% for patients with intranodal growth (12). VC related mortality usually results from failure to control the disease once it has progressed beyond the site of origin. In these patients diagnosis is often delayed by the patient or physician (6).

For patients with a local recurrence disease-specific survival decreases from 90% to 69% (35). Furthermore, disease-specific survival is worse for patients who develop a local recurrence within two years compared to patients that develop a local recurrence more than two years after primary treatment (53% versus 76%) (35, 36).

The majority of groin recurrences (~ 70%) develop within the first year after primary treatment, with a median time until recurrence of 7 months (35, 37, 38). Prognosis for patients with a groin recurrence is very poor. Most patients die of disease within two years after development of the groin recurrence (25, 34, 35, 38). On the contrary, a recently published study found an overall survival rate of 50% for 30 patients with a groin recurrence after 7 years. Especially patients who received multimodal treatment for their groin recurrence performed better (39).

Local recurrence

Recurrent disease is an important clinical challenge in the treatment of VC. Despite all developments in treatment strategies, recurrence rates of VC are still high: 12-37% of VC patients develop a recurrence (25, 26, 40) of which 50% are local (25, 26, 37, 40). There are several known risk factors for a local recurrence. The width of the tumour free margin is considered the most important predictive factor for local recurrences. It is known that tumour-positive margins are associated with recurrence and poor prognosis. The minimal safe tumour-free margin is one of the most relevant clinical questions in the primary surgical treatment of VC, especially given the treatment-related morbidity associated with radical surgery in the genital area. Most current guidelines advise a minimal tumour-free margin of 8 mm (27-30) which is based on a study by Heaps et al. The authors found that patients with a tumour-free margin of ≥ 8 mm did not develop a local recurrence (41). However, other studies on the tumour-free margin distance report contradictory results (18, 42-49). Another strong prognostic factor is tumour positive lymph node(s) (9, 25, 35, 40, 50). Intriguingly, tumour positive lymph nodes increase the chance of a groin recurrence as well as the chance of a local recurrence (50). This might be explained by a biological more aggressive tumour behaviour if lymph node metastases are present. Also the number of tumour positive lymph nodes (9), the size of nodal metastases and the presence of extranodal growth and the number of removed lymph nodes during IFL are known prognostic factors (6, 40). Other risk factors for recurrent disease are higher age (40, 50), greater tumour size (25, 50), depth of invasion of > 2 mm (40, 50) and lymph vascular space invasion (LVSI) (37, 40).

Pathogenesis of vulvar cancer

The pathogenesis of VC can typically be sub classified into HPV-independent and HPV-dependent VC (3, 9, 40, 51, 52). These two different types of VC have different epidemiological, clinical, pathological and molecular characteristics and it becomes more and more clear that both tumour types should be considered as two separate entities (3).

HPV-independent VC account for around 70% of all VC, usually occur in older patients and are associated with lichen sclerosus (LS) and mutations in *TP53*. The presumed precursor lesion in this type of VC is differentiated vulvar intraepithelial neoplasia (dVIN) (3, 51, 53). The exact mechanisms involved in the progression from LS and dVIN into VC are currently unclear.

HPV-dependent VC account for around 30% of all VC and have vulvar high grade squamous intraepithelial lesions (HSIL) as a precursor lesion (2, 3). The most prevalent HPV-types found in these VCs are HPV 16 in 60-78% of the cases, followed by HPV 18 in 5-16% of the cases (9, 54-60). Other encountered HPV types are HPV 31, 33 and 45 (3). This tumour type is more common in younger patients (35-65 years) and is associated with smoking, a higher number of sexual partners, and a compromised immune status (3, 9, 51).

Although HPV-independent and HPV-dependent VC are pathologically distinct, the clinical relevance of this distinction has not yet been established. In another tumour type with a similar dualistic classification, head and neck squamous cell carcinomas (HNSCC), the HPV presence has proven to improve prognosis. In addition, HPV-dependent HNSCC show a better response to adjuvant therapy (61-63). In VC, the prognostic significance of HPV on survival has been debated and is not yet fully understood (54). There is some suggestion that HPV-dependent VC, similar to HPV-dependent HNSCC, have a more favourable prognosis compared to HPV-independent VC (55-58, 60). However, other studies could not confirm this prognostic advantage (3, 54, 59, 64).

Vulvar pre-malignancies

About 50-80% of VC patients present with an epithelial disorder adjacent to the VC (3, 65, 66). Most VCs originate in these intraepithelial lesions, which precede the development of invasive disease by a variable period of time (3). The most recent classification system of the International Society for the Study of Vulvovaginal Disease (ISSVD) distinguishes between the HPV-independent precursor lesion dVIN (Figure 3a) and the HPV-dependent lesion HSIL (formerly known as usual VIN) (Figure 3b). The characteristics of these vulvar pre-malignancies are described in table 2.

Table 2: vulvar precursor lesions

	dVIN	HSIL
Synonym	VIN, differentiated type	Usual type VIN or VIN 2/3
HPV status	Negative	Positive (HPV 16-18)
Proportion	2-10%	± 90%
Characteristics	Older women	Younger women
	LS related	Smoking related
	TP53 mutations	Promiscuity
	Often adjacent to VC	Compromised immunity
		Often multifocal
Progression rate	±80% if untreated	9-16% if untreated

VIN: vulvar intraepithelial neoplasia
HSIL: high grade squamous intraepithelial lesion
HPV: human papilloma virus
LS: lichen sclerosus

Molecular features

More detailed information on the molecular background of VC and specifically information on genetic and epigenetic changes can provide valuable insight in the pathogenesis of VC. Previous studies on different types of cancer have shown that genetic and epigenetic alteration status can help in predicting prognosis and guide targeted therapy (67-71). Malignant transformation is determined by a sequence of genetic and epigenetic events often involving dysregulation of the cell cycle control. Cell cycle alterations are mainly caused by alterations in the p53 or pRb (p16/pRb/cyclin-D1) pathways. P53 overexpression is found in 40-81% and *Tp53* mutations in 20-30% of the VC patients and is unrelated to HPV-infection. The pRb pathway is mediated by inactivation of Rb through its phosphorylation. The P16 protein can act as an inhibitor by preventing this phosphorylation. Loss of cell cycle control via this pathway is thus caused by somatic mutations in Rb or by disrupted p16 function through somatic mutations or promoter hypermethylation. Promoter hypermethylation of p16 is common and this gene is currently considered the most frequently inactivated tumour suppressor gene in cancer (72, 73).

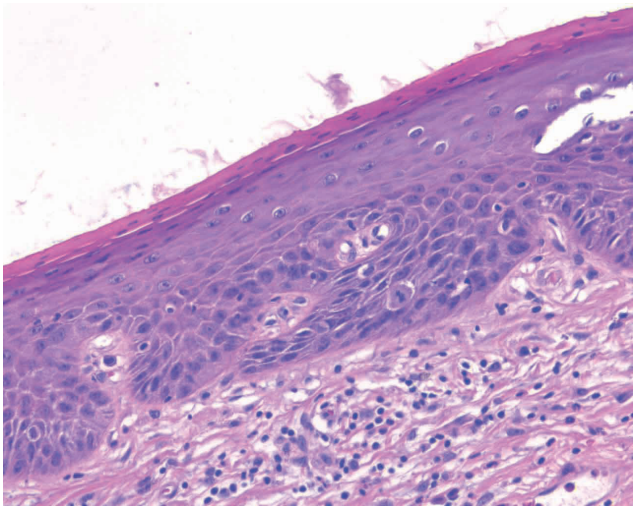


Figure 3a: Differentiated vulvar intraepithelial neoplasia (dVIN)

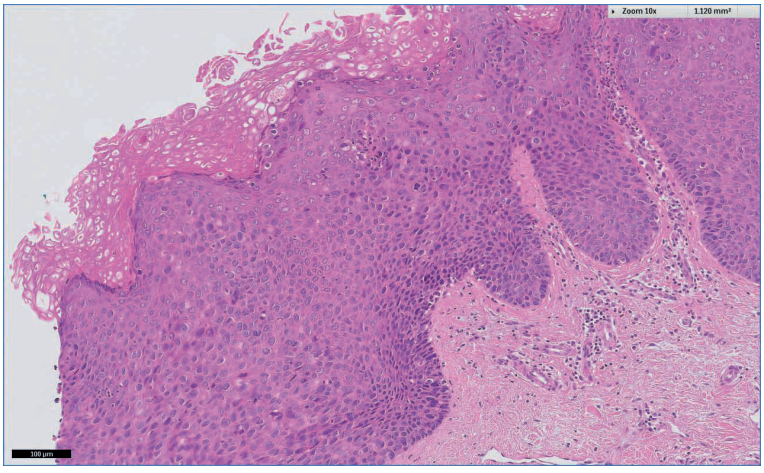


Figure 3b: Vulvar high grade squamous intra-epithelial lesion (HSIL)

At this moment, most is known about the molecular mechanisms involved in the development of vulvar HSIL and HPV-dependent VC (3, 74). This knowledge is partially acquired due to the great similarities with cervical cancer in which the role of HPV has been studied extensively (75). In HPV-dependent VC, the immune system fails to produce an effective response to high-risk HPV. This leads to virus persistence and integration and replication of the viral DNA in epidermal cells (75). The longer the infection persists, the longer the viral oncoproteins E6 and E7 can interfere with important cell cycle control mechanisms, which will lead to escape from programmed cell death and transformation (52, 75-77). E6 degrades the tumour suppressor p53, which leads to absence of cell cycle arrest. E7 inactivates the retinoblastoma tumour suppressor gene product, which results in hyperproliferation of tumour cells and overexpression of p16 and p14 (3, 76, 77). As a result, p16 has proven to be an excellent surrogate marker for high risk HPV infection.

HPV-independent VCs have been much less studied and the molecular mechanisms involved in its development have not yet been fully elucidated. Somatic mutations in *TP53*, leading to an aberrant function of the p53 protein, have been detected in a high percentage of HPV-independent VC and dVIN and seem to have an important function in the pathogenesis of VC (52, 76-79). Because aberrant p53 expression has also been described in precursor lesions of the vulva, this may be an initiating event in vulvar carcinogenesis (53). This is supported by a study by Rolfe et al. in which a *TP53* mutational analysis identified an identical genotype in the adjacent precursor lesion in 50% of the VC patients (n=27) (78). Studies on somatic mutations in VC other than in the *TP53* gene are limited. Holway et al. studied eight vulvar cancer patients and identified *PTEN* mutations in five of eight vulvar cancer patients, suggesting that *PTEN* is frequently altered in VCs (79). In a study on 108 VC samples published by Trietsch et al., somatic mutations were found in *CDKN2A* (13%), *HRAS* (9%), *PIK3CA* (7%) and *PP2R1A* (3%) (80).

Future research can further elucidate the molecular features involved in the pathogenesis of VC. The current developments in molecular diagnostics and especially (epi)genetic testing will provide a substantial contribution to our knowledge on this pathogenesis, in particular on the HPV-independent VC. At this moment it is unclear whether the different types of VC indeed represent a difference in clinical behaviour and thus whether this subdivision has clinical relevance. Differences in clinical behaviour might cause a change in treatment strategy of VC patients. Gaining knowledge of the pathogenesis will contribute to the development of a more individualized treatment strategy for VC patients.

Thesis aim and outline

The objectives of this thesis can be subdivided into clinical questions and questions regarding the pathogenesis of vulvar cancer. The overarching theme however is to use these data to improve and personalise the treatment of patients with vulvar cancer. The clinical section is covered in chapter 2-4. **Chapter 2** reports on the influence of the histological margin distance and local recurrence rate. In this study we combine the results of a meta-analysis of the currently available literature with a retrospective cohort study in the LUMC. **Chapter 3** describes the clinical outcome of vulvar cancer patients treated for groin lymph node metastasis, comparing extensive inguinofemoral lymphadenectomy with debulking of enlarged lymphnodes. **Chapter 4** presents a review on recurrent VC and literature concerning treatment of recurrent VC.

The second section of this thesis (chapter 5-7) is devoted to work that intends to improve our molecular understanding and diagnosis of vulvar (pre)cancers. It starts, in **chapter 5**, with a review on the (epi)genetic alterations in VC and its precursor lesions described in the current literature. In **chapter 6** we investigated whether stathmin immunostaining can be used in the differential diagnosis of vulvar precancerous lesions. In **chapter 7** a comprehensive genetic landscape of a large series of vulvar precursor lesions and VC is presented, including the clinical relevance.

The general discussion in **chapter 8** gives an overview of the findings presented in this thesis and a glance at future perspectives in the developments in treatment of VC and insight of the pathogenesis of VC.

References

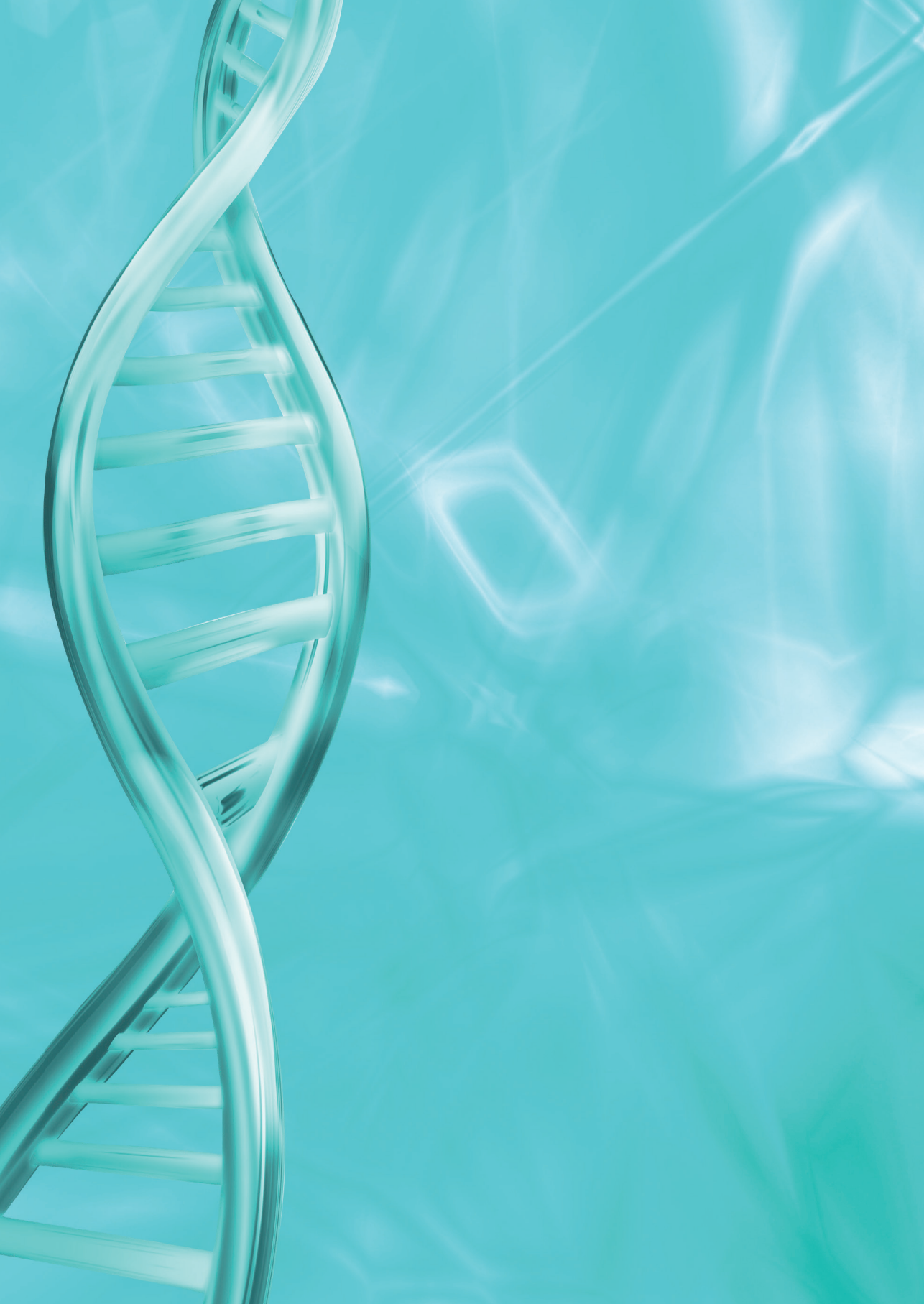
1. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.
2. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. Crit RevOncolHematol. 2008;68(2):131-56.
3. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. Histopathology. 2013;62(1):161-75.
4. van de Nieuwenhof HP, Massuger LF, van der Avoort IA, Bekkers RL, Casparie M, Abma W, et al. Vulvar squamous cell carcinoma development after diagnosis of VIN increases with age. EurJCancer. 2009;45(5):851-6.
5. Schuurman MS, van den Einden LC, Massuger LF, Kiemeney LA, van der Aa MA, de Hullu JA. Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma. EurJCancer. 2013;49(18):3872-80.
6. Homesley HD. Management of vulvar cancer. Cancer. 1995;76(10 Suppl):2159-70.
7. IKNL: cijfers over kanker 2016 [Available from: http://www.cijfersoverkanker.nl/selecties/Dataset_1/img5742c1b22536e.
8. de Hullu JA, van der Zee AG. Surgery and radiotherapy in vulvar cancer. Crit RevOncolHematol. 2006;60(1):38-58.
9. Canavan TP, Cohen D. Vulvar cancer. AmFamPhysician. 2002;66(7):1269-74.
10. Velden vd, Fons G, Lawrie TA. Primary groin irradiation versus primary groin surgery for early vulvar cancer. CochraneDatabaseSystRev. 2011(5):CD002224.
11. Oncology FCoG. FIGO staging for carcinoma of the vulva, cervix, and corpus uteri. Int J Gynaecol Obstet. 2014;125(2):97-8.
12. van der Steen S, de Nieuwenhof HP, Massuger L, Bulten J, de Hullu JA. New FIGO staging system of vulvar cancer indeed provides a better reflection of prognosis. GynecolOncol. 2010;119(3):520-5.
13. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
14. Tabbaa ZM, Gonzalez J, Sznurkowski JJ, Weaver AL, Mariani A, Cliby WA. Impact of the new FIGO 2009 staging classification for vulvar cancer on prognosis and stage distribution. GynecolOncol. 2012;127(1):147-52.
15. Gadducci A, Cionini L, Romanini A, Fanucchi A, Genazzani AR. Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer. Crit RevOncolHematol. 2006;60(3):227-41.
16. de Hullu JA, van der Zee AG. Groin surgery and the sentinel lymph node. BestPractResClinObstetGynaecol. 2003;17(4):571-89.
17. Gaarenstroom KN, Kenter GG, Trimbos JB, Agous I, Amant F, Peters AA, et al. Postoperative complications after vulvectomy and inguinofemoral lymphadenectomy using separate groin incisions. IntJGynecolCancer. 2003;13(4):522-7.

18. Rouzier R, Haddad B, Plantier F, Dubois P, Pelisse M, Paniel BJ. Local relapse in patients treated for squamous cell vulvar carcinoma: incidence and prognostic value. Obstet Gynecol. 2002;100(6):1159-67.
19. van der Zee AG, Oonk MH, de Hullu JA, Ansink AC, Vergote I, Verheijen RH, et al. Sentinel node dissection is safe in the treatment of early-stage vulvar cancer. JClinOncol. 2008;26(6):884-9.
20. Levenback CF, Ali S, Coleman RL, Gold MA, Fowler JM, Judson PL, et al. Lymphatic mapping and sentinel lymph node biopsy in women with squamous cell carcinoma of the vulva: a gynecologic oncology group study. JClinOncol. 2012;30(31):3786-91.
21. Velden vd. Surgical interventions for early squamous cell carcinoma of the vulva. Cochrane Database of Systematic Reviews. 2000.
22. Berek JS, Hacker N.F. Berek and Hacker's Gynecologic Oncology. 2014;6 ed Philadelphia LWW.
23. Hyde SE, Valmadre S, Hacker NF, Schilthuis MS, Grant PT, van d, V. Squamous cell carcinoma of the vulva with bulky positive groin nodes-nodal debulking versus full groin dissection prior to radiation therapy. IntJGynecolCancer. 2007;17(1):154-8.
24. Hinten F, van den Einden LC, Hendriks JC, van der Zee AG, Bulten J, Massuger LF, et al. Risk factors for short- and long-term complications after groin surgery in vulvar cancer. BrJCancer. 2011;105(9):1279-87.
25. Coulter J, Gleeson N. Local and regional recurrence of vulval cancer: management dilemmas. Best Pract Res Clin Obstet Gynaecol. 2003;17(4):663-81.
26. Salom EM, Penalver M. Recurrent vulvar cancer. Curr TreatOptions Oncol. 2002;3(2):143-53.
27. National Dutch Guideline Gynaecologic Tumors; Vulvar Carcinoma. http://www.oncoline.nl ed2015.
28. RCOG guideline vulval carcinoma. https://www.rcog.org.uk/globalassets/documents/guidelines/vulvalcancerguideline.pdf ed2015.
29. National Comprehensive Cancer Network guideline on vulvar cancer. https://www.nccn.org/store/login/login.aspx?ReturnURL=http://www.nccn.org/professionals/physician_gls/pdf/vulvar.pdf ed2016.
30. Guideline vulvar cancer, national cancer institute. 2015.
31. Faul CM, Mirmow D, Huang Q, Gerszten K, Day R, Jones MW. Adjuvant radiation for vulvar carcinoma: improved local control. Int J RadiatOncol BiolPhys. 1997;38(2):381-9.
32. Mahner S, Jueckstock J, Hilpert F, Neuser P, Harter P, de Gregorio N, et al. Adjuvant therapy in lymph node-positive vulvar cancer: the AGO-CaRE-1 study. J Natl Cancer Inst. 2015;107(3).
33. Homesley HD, Bundy BN, Sedlis A, Adcock L. Radiation therapy versus pelvic node resection for carcinoma of the vulva with positive groin nodes. Obstet Gynecol. 1986;68(6):733-40.
34. Gonzalez BJ, Magrina JF, Gaffey TA, Hernandez JL, Webb MJ, Cliby WA, et al. Long-term survival and disease recurrence in patients with primary squamous cell carcinoma of the vulva. GynecolOncol. 2005;97(3):828-33.
35. Te Grootenhuis NC, van der Zee AG, van Doorn HC, van d, V, Vergote I, Zanagnolo V, et al. Sentinel nodes in vulvar cancer: Long-term follow-up of the GROningen INternational Study on Sentinel nodes in Vulvar cancer (GROINSS-V) I. GynecolOncol. 2016;140(1):8-14.

36. Ragupathy K, Grandidge L, Strelley K, Wang H, Tidy J. Early and late vulval cancer recurrences: Are they different? J Obstet Gynaecol. 2016;36(4):518-21.
37. Maggino T, Landoni F, Sartori E, Zola P, Gadducci A, Alessi C, et al. Patterns of recurrence in patients with squamous cell carcinoma of the vulva. A multicenter CTF Study. Cancer. 2000;89(1):116-22.
38. Stehman FB, Bundy BN, Ball H, Clarke-Pearson DL. Sites of failure and times to failure in carcinoma of the vulva treated conservatively: a Gynecologic Oncology Group study. AmJObstetGynecol. 1996;174(4):1128-32.
39. Frey JN, Hampl M, Mueller MD, Gunthert AR. Should Groin Recurrence Still Be Considered as a Palliative Situation in Vulvar Cancer Patients?: A Brief Report. Int J Gynecol Cancer. 2016;26(3):575-9.
40. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
41. Heaps JM, Fu YS, Montz FJ, Hacker NF, Berek JS. Surgical-pathologic variables predictive of local recurrence in squamous cell carcinoma of the vulva. Gynecol Oncol. 1990;38(3):309-14.
42. Chan JK, Sugiyama V, Pham H, Gu M, Rutgers J, Osann K, et al. Margin distance and other clinico-pathologic prognostic factors in vulvar carcinoma: a multivariate analysis. Gynecol Oncol. 2007;104(3):636-41.
43. Viswanathan AN, Pinto AP, Schultz D, Berkowitz R, Crum CP. Relationship of margin status and radiation dose to recurrence in post-operative vulvar carcinoma. Gynecol Oncol. 2013;130(3):545-9.
44. Tantipalakorn C, Robertson G, Marsden DE, Gebski V, Hacker NF. Outcome and patterns of recurrence for International Federation of Gynecology and Obstetrics (FIGO) stages I and II squamous cell vulvar cancer. ObstetGynecol. 2009;113(4):895-901.
45. Iacoponi S, Zapardiel I, Diestro MD, Hernandez A, De SJ. Prognostic factors associated with local recurrence in squamous cell carcinoma of the vulva. J Gynecol Oncol. 2013;24(3):242-8.
46. de Hullu JA, Hollema H, Lolkema S, Boezen M, Boonstra H, Burger MP, et al. Vulvar carcinoma. The price of less radical surgery. Cancer. 2002;95(11):2331-8.
47. Baiocchi G, Mantoan H, de Brot L, Badiglian-Filho L, Kumagai LY, Faloppa CC, et al. How important is the pathological margin distance in vulvar cancer? Eur J Surg Oncol. 2015;41(12):1653-8.
48. Groenen SM, Timmers PJ, Burger CW. Recurrence rate in vulvar carcinoma in relation to pathological margin distance. Int J Gynecol Cancer. 2010;20(5):869-73.
49. Woelber L, Choschzick M, Eulenburg C, Hager M, Jaenicke F, Gieseking F, et al. Prognostic value of pathological resection margin distance in squamous cell cancer of the vulva. Ann Surg Oncol. 2011;18(13):3811-8.
50. Woelber L, Mahner S, Voelker K, Eulenburg CZ, Gieseking F, Choschzick M, et al. Clinicopathological prognostic factors and patterns of recurrence in vulvar cancer. Anticancer Res. 2009;29(2):545-52.
51. van der Avoort IA, Shirango H, Hoevenaars BM, Grefte JM, de Hullu JA, de Wilde PC, et al. Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways. IntJGynecolPathol. 2006;25(1):22-9.

52. Preti M, Scurry J, Marchitelli CE, Micheletti L. Vulvar intraepithelial neoplasia. BestPractResClinObstetGynaecol. 2014;28(7):1051-62.
53. Hantschmann P, Sterzer S, Jeschke U, Friese K. P53 expression in vulvar carcinoma, vulvar intraepithelial neoplasia, squamous cell hyperplasia and lichen sclerosus. Anticancer Res. 2005;25(3A):1739-45.
54. Alonso I, Fuste V, Del PM, Castillo P, Torne A, Fuste P, et al. Does human papillomavirus infection imply a different prognosis in vulvar squamous cell carcinoma? GynecolOncol. 2011;122(3):509-14.
55. Lindell G, Nasman A, Jonsson C, Ehrsson RJ, Jacobsson H, Danielsson KG, et al. Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node. GynecolOncol. 2010;117(2):312-6.
56. Ansink AC, Krul MR, De Weger RA, Kleyne JA, Pijpers H, Van TH, et al. Human papillomavirus, lichen sclerosus, and squamous cell carcinoma of the vulva: detection and prognostic significance. GynecolOncol. 1994;52(2):180-4.
57. Larsson GL, Helenius G, Andersson S, Elgh F, Sorbe B, Karlsson MG. Human papillomavirus (HPV) and HPV 16-variant distribution in vulvar squamous cell carcinoma in Sweden. Int J Gynecol Cancer. 2012;22(8):1413-9.
58. Monk BJ, Burger RA, Lin F, Parham G, Vasilev SA, Wilczynski SP. Prognostic significance of human papillomavirus DNA in vulvar carcinoma. ObstetGynecol. 1995;85(5 Pt 1):709-15.
59. Pinto AP, Schlecht NF, Pintos J, Kaiano J, Franco EL, Crum CP, et al. Prognostic significance of lymph node variables and human papillomavirus DNA in invasive vulvar carcinoma. Gynecol Oncol. 2004;92(3):856-65.
60. van de Nieuwenhof HP, van Kempen LC, de Hullu JA, Bekkers RL, Bulten J, Melchers WJ, et al. The etiologic role of HPV in vulvar squamous cell carcinoma fine tuned. Cancer Epidemiol Biomarkers Prev. 2009;18(7):2061-7.
61. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. ClinCancer Res. 2009;15(22):6758-62.
62. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. JNatlCancer Inst. 2008;100(4):261-9.
63. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. IntJCancer. 2007;121(8):1813-20.
64. Choschzick M, Hantaredja W, Tennstedt P, Gieseking F, Wolber L, Simon R. Role of TP53 mutations in vulvar carcinomas. Int J Gynecol Pathol. 2011;30(5):497-504.
65. Rouzier R, Morice P, Haie-Meder C, Lhomme C, Avril MF, Duvillard P, et al. Prognostic significance of epithelial disorders adjacent to invasive vulvar carcinomas. GynecolOncol. 2001;81(3):414-9.
66. Kokka F, Singh N, Faruqi A, Gibbon K, Rosenthal AN. Is differentiated vulval intraepithelial neoplasia the precursor lesion of human papillomavirus-negative vulval squamous cell carcinoma? IntJGynecolCancer. 2011;21(7):1297-305.
67. Santarpia L, Qi Y, Stemke-Hale K, Wang B, Young EJ, Booser DJ, et al. Mutation profiling identifies numerous rare drug targets and distinct mutation patterns in different clinical subtypes of breast cancers. Breast Cancer ResTreat. 2012;134(1):333-43.

68. Stelloo E, Nout RA, Osse EM, Jurgenliemk-Schulz IJ, Jobsen JJ, Lutgens LC, et al. Improved risk assessment by integrating molecular and clinicopathological factors in early-stage endometrial cancer - combined analysis of PORTEC cohorts. Clin Cancer Res. 2016.
69. Schneider BJ, Kalemkerian GP. Personalized Therapy of Small Cell Lung Cancer. Adv Exp Med Biol. 2016;890:149-74.
70. Feldman R, Gatalica Z, Knezetic J, Reddy S, Nathan CA, Javadi N, et al. Molecular profiling of head and neck squamous cell carcinoma. Head Neck. 2016;38 Suppl 1:E1625-38.
71. Wegman P, Ahlin C, Sorbe B. Genetic alterations in the K-Ras gene influence the prognosis in patients with cervical cancer treated by radiotherapy. IntJGynecolCancer. 2011;21(1):86-91.
72. Lerma E, Esteller M, Herman JG, Prat J. Alterations of the p16/Rb/cyclin-D1 pathway in vulvar carcinoma, vulvar intraepithelial neoplasia, and lichen sclerosus. HumPathol. 2002;33(11):1120-5.
73. Knopp S, Trope C, Nesland JM, Holm R. A review of molecular pathological markers in vulvar carcinoma: lack of application in clinical practice. JClinPathol. 2009;62(3):212-8.
74. Rakislova N, Saco A, Sierra A, Del Pino M, Ordi J. Role of Human Papillomavirus in Vulvar Cancer. Adv Anat Pathol. 2017;24(4):201-14.
75. Crosbie EJ, Einstein MH, Franceschi S, Kitchener HC. Human papillomavirus and cervical cancer. Lancet. 2013;382(9895):889-99.
76. Ueda Y, Enomoto T, Kimura T, Yoshino K, Fujita M, Kimura T. Two distinct pathways to development of squamous cell carcinoma of the vulva. JSkin Cancer. 2011;2011:951250.
77. Leonard B, Kridelka F, Delbecque K, Goffin F, Demoulin S, Doyen J, et al. A clinical and pathological overview of vulvar condyloma acuminatum, intraepithelial neoplasia, and squamous cell carcinoma. BiomedResInt. 2014;2014:480573.
78. Rolfe KJ, MacLean AB, Crow JC, Benjamin E, Reid WM, Perrett CW. TP53 mutations in vulval lichen sclerosus adjacent to squamous cell carcinoma of the vulva. BrJCancer. 2003;89(12):2249-53.
79. Holway AH, Rieger-Christ KM, Miner WR, Cain JW, Dugan JM, Pezza JA, et al. Somatic mutation of PTEN in vulvar cancer. ClinCancer Res. 2000;6(8):3228-35.
80. Trietsch MD, Spaans VM, ter Haar NT, Osse EM, Peters AA, Gaarenstroom KN, et al. CDKN2A(p16) and HRAS are frequently mutated in vulvar squamous cell carcinoma. GynecolOncol. 2014;135(1):149-55.



CHAPTER 2

Tumour-free margins in vulvar squamous cell carcinoma: does distance really matter?

Linda S. Nooij
Michelle A. van der Slot
Olaf M. Dekkers
Theo Stijnen
Katja N. Gaarenstroom
Carien L. Creutzberg
Vincent T.H.B.M. Smit
Tjalling Bosse
Mariette I.E. van Poelgeest

European Journal of Cancer 2016;65:139-149

Abstract

Background: There is no consensus on the width of tumour-free margins after surgery for vulvar squamous cell carcinoma (VSCC). Most current guidelines recommend tumour-free margins of ≥8 mm. The aim of this study was to investigate whether a margin of <8 mm is associated with an increased risk of local recurrence in VSCC.

Methods: A meta-analysis of the available literature and a cohort study of 148 VSCC patients seen at a referral centre from 2000 to 2012 was performed. The primary end-point of the cohort study was a histologically confirmed ipsilateral local recurrence within 2 years after primary treatment in relation to the margin distance.

Results: Based on 10 studies, the meta-analysis showed that a tumour-free margin of <8 mm is associated with a higher risk of local recurrence compared to a tumour-free margin of ≥8 mm (pooled risk ratio, 1.99 [95% confidence interval {CI}: 1.13–3.51], p=0.02). In the cohort study, we found no clear difference in the risk of local recurrence in the <8 versus ≥8 mm group; however, 40% of the patients in the <8 mm group received additional treatment. Tumour-positive margin was the only independent risk factor for local recurrence in the multivariable analysis (hazard ratio, 0.21 [95% CI 0.08–0.55]).

Conclusions: This work provides important data to question the commonly used 8 mm margin as a prognosticator for local recurrence. More research is needed to address the question of whether additional treatment improves the prognosis in patients with a tumour-free margin of < 8 mm.

Introduction

The fundamental goal of curative oncological surgery is complete tumour resection (1, 2). Tumour-positive margins, usually expressed in millimetres of distance from the tumour to the nearest line of resection, are strongly associated with recurrence and poor prognosis (2-5). The minimal safe tumour-free margin is an important clinical issue in several tumour types where tissue-sparing surgery is desired (e.g., head and neck squamous cell carcinomas, breast cancer, soft tissue sarcomas, and penile cancer) (2, 6-10). The definition of a minimal safe tumour-free margin varies between 1–10 mm for different tumour types (2, 8, 9). Level one evidence is not available, and consensus or guidelines on the optimal tumour-free margin for many tumours are lacking (2, 11-14). Nonetheless, important clinical decisions are based on these tumour-free margins including the need for additional treatment (re-excision or (chemo)radiotherapy), which is associated with additional discomfort for patients, treatment-related morbidity, and increased health care costs (4, 7, 14-16).

Vulvar cancer is a rare malignancy, accounting for around 5% of all gynaecological cancers, with squamous cell carcinoma as the most common histologic subtype (17, 18). Surgery is the treatment of choice for most patients, but can lead to significant morbidity when the tumour is near the clitoris, urethra, or anus (3, 19). Patients with vulvar squamous cell carcinoma (VSCC) are at high risk for developing local recurrent disease. Approximately 25% of patients experience a local recurrence after primary treatment (20, 21). Although most local recurrences develop within 2 years, late "recurrences" often occur in VSCC as shown in a recent long-term follow-up study that found an overall local recurrence risk of 27.2% after 5 years, and 39.5% after 10 years (22). Local recurrences are considered the result of residual tumour cells after inadequate surgical margins and arise around the surgical scar. Late recurrences are unlikely to arise from residual tumour cells after inadequate resection, and are better defined as second primary tumours. Second primary tumours in VSCC arise from a persistent precancerous field, which encompasses altered cells with high premalignant potential (14, 23, 24). In VSCC, both human papillomavirus (HPV) (high-grade squamous intraepithelial lesion) and non-HPV (differentiated vulvar intraepithelial neoplasia) related precancerous lesions have been defined and are frequently identified surrounding VSCC (18, 25). This so-called "field effect" is considered to be responsible for the increased risk of the developing second primary tumours in patients with VSCC (24).

Given the treatment-related morbidity associated with radical surgery in the genital area, the minimal safe tumour-free margin is one of the most relevant clinical questions in the primary surgical treatment of VSCC. Additional treatment is generally advised when the tumour-free margin (i.e. the histological margin after fixation) is involved or close,

but a uniform definition for "close margin" is lacking (12, 13, 26). The Royal College of Obstetricians & Gynaecologist guidelines on the surgical treatment of VSCC (27) advises a minimal tumour-free margin of 10 mm, while the Dutch and the American National Cancer institute and National Comprehensive Cancer Network guidelines recommend a minimal tumour-free resection margin of 8 mm (12, 13, 26). To reach this, a surgical margin of 1-2 cm around the tumour is recommended. These guidelines are based upon relatively small studies (3-5, 19, 28-32). Additionally, it is not clear if additional treatment reduces the risk for local recurrence in VSCC (33).

The aim of this study was to investigate whether a tumour-free margin <8 mm is associated with local recurrence after primary surgery for VSCC. For this purpose, a systematic review and meta-analysis of the available literature was performed. Additionally, a large cohort study was conducted at a referral centre for patients with VSCC.

Methods

I. Systematic review and meta-analysis

Search eligibility and search strategy

A systematic review of the literature on the tumour-free margin status related to risk of recurrence in VSCC was performed. Relevant studies were identified from a literature search of PubMed, Embase, Web of Science, Cochrane database, and ScienceDirect. The search was conducted in October 2015. A combination of Medical Subject Headings and free text words were formulated after consulting a medical librarian. Our search included the terms vulvar neoplasm, vulva(r) carcinoma, surgical margin, histo(patho) logical margin, clinical margin, excision margin or margin (Appendix A). Studies on local recurrence risk in relation to the tumour-free margin in VSCC were eligible for inclusion. Exclusion criteria were languages other than English, Dutch, German, French, or Italian. Studies that compared local recurrence risk for patients with tumour-positive margins with tumour-free margins were also excluded because we focused on comparison of close versus wide margins. All articles were assessed based on the title, abstract, or full article. The electronic search was complemented with a manual search of references from relevant articles.

Data extraction and risk of bias assessment

For all studies, we extracted the following data: number of included patients, definition of local recurrence, number of local recurrences, and additional treatment (including reexcisions and radiotherapy). Two articles that reported on a tumour-free margin of 1 cm were also included. Three studies that only reported data on a smaller tumour-free margin (3 or 5 mm) were excluded because our focus was a tumour-free margin of 8

mm. When possible, patients with a tumour-positive margin were analysed as a separate patient group. The number of local recurrences was based on the definitions used in included articles. A risk of bias analysis was performed. All studies were evaluated for selection, performance, attrition, detection, and reporting bias according to the 'methods guide for comparative effectiveness reviews' (34).

II. Cohort study

Patient and tumour characteristics

A cohort study was performed of consecutive patients who were surgically treated for primary VSCC between 2000 and 2012 in the Leiden University Medical Centre. Histological slides were collected from the pathology archive, and patient characteristics were gathered from electronic patient charts after approval by the institutional review board.

All gross specimens were handled according to the local protocol, and minimal tumour-free margins were measured on haematoxylin and eosin stained slides from formalin-fixed, paraffin-embedded tissue blocks. To assure uniform assessment, minimal margin measurements were revised by an expert gynaecopathologist (TB) blinded to the patient's recurrence status. For this revision, slides were scanned with the Philips Ultra-Fast Scanner, and the Philips Digital Pathology Solutions software was used to measure the histological margins using a digital ruler. The tumour-free margin was defined as the closest distance from the invasive tumour to the lateral or basal resection margin.

Surgical treatment of the vulva consisted of a vulvectomy (removal of part or all of the tissues from the vulva; i.e. labia majora, labia minora, and the clitoris) or wide local excision (removal of the tumour with a macroscopic margin of at least 1 cm). Additional treatment was generally started within 6 weeks after the primary surgery and consisted of reexcision or radiotherapy. Additional treatment was recommended for patients with tumour-positive margins and was considered for patients with a tumour-free margin <8 mm who had other risk factors (advanced tumour stage, positive lymph nodes, or lymphovascular space invasion). All patients were discussed in a multidisciplinary meeting. When considered feasible, a reexcision was performed. Otherwise, the patient received additional radiotherapy comprising a total dose of 50.4 Gy in fractions of 1.8 Gy, with five fractions per week administered. Follow-up consisted of outpatient visits every 2–3 months during the first 2 years after treatment, every 4–6 months during the third and fourth years, and annually thereafter.

Definition of local recurrence

We defined a local recurrence as a histologically confirmed recurrence of VSCC within 2 years that was located on the ipsilateral side of the vulva as the primary tumour. A 2-year period after primary treatment was chosen because up to 80% of all local recurrences of VSCC occur within this time period after the initial treatment (5, 21, 22, 28, 29, 35). A new tumour developing more than 2 years after primary treatment and/or on the contralateral side of the vulva was considered a second primary tumour.

Statistical analysis

Statistical analysis for the meta-analysis was performed using Review Manager 5.3. A random effects analysis was carried out to estimate the pooled risk ratio for the association between the tumour-free margin (<8 mm versus ≥8 mm) and local recurrence risk.

For the cohort study, statistical analysis was performed with SPSS version 20.0. We divided the patients into groups with a tumour-positive margin and a tumour-free margin of <8 mm and ≥8 mm. The chi-square test was used to compare baseline characteristics between groups. A competing risk analysis (accounting for death as a competing risk) was performed to estimate local recurrence risk. In a post hoc analysis, local recurrence risk was also determined for other tumour-free margin cutoff values (2, 4, and 6 mm). Univariable and multivariable analyses were performed with the Cox proportional hazard model. Multivariable analysis included all variables with a p-value <0.1 in the univariable analysis because these variables were considered important factors for the probability of developing a recurrence.

Results

Meta-analysis

A total of 368 articles were identified through an electronic literature search. Seven articles were added through a complementary manual search for articles. Based on the title of the article, 292 articles were excluded. From the remaining 83 articles, the abstract was reviewed, after which another 43 articles were excluded. Ten cohort studies published between 1990 and 2015 investigating the association between tumour-free margin and local recurrence risk were included (Figure 1) (3-5, 19, 28-32, 36). The range of included patients was 79–205, and the mean follow-up time ranged from 31 to 110 months. The risk of bias analysis did not reveal any major bias in the included studies, although in most articles, the evaluated biases were not described.

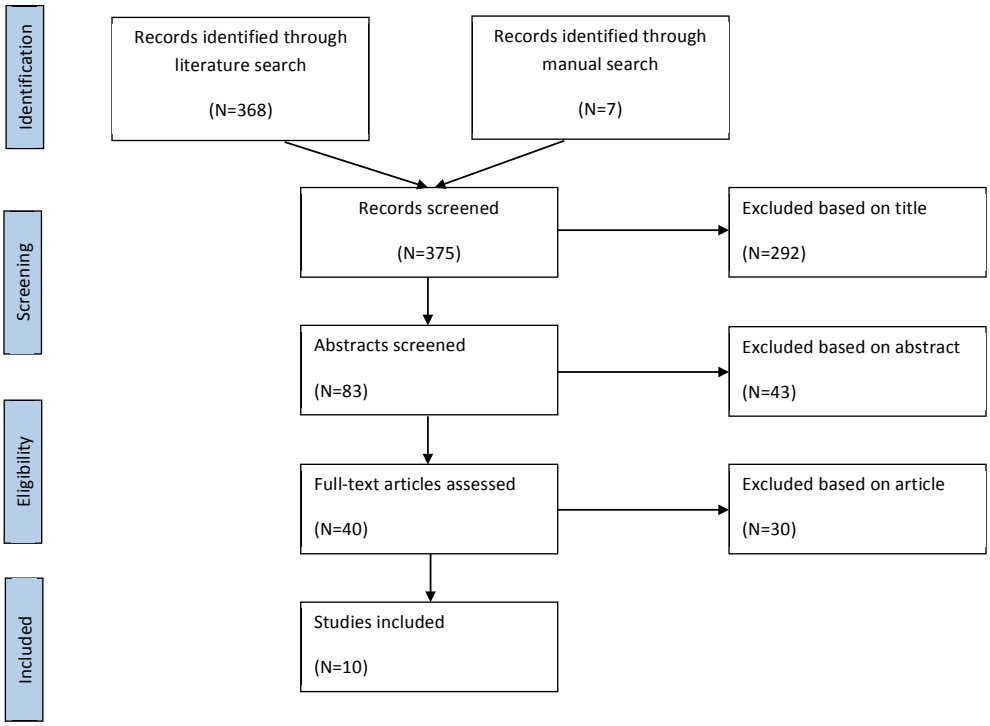


Figure 1: Flowchart illustrating inclusion and exclusion of articles for the meta-analysis

Eight studies compared local recurrence risk for patients with a tumour-free margin of <8 mm with a tumour-free margin of ≥8 mm. Two studies compared a tumour-free margin of <1 cm with ≥1 cm. Study descriptions are summarised in table 1 and the risk of bias analysis in supplementary table 1. Due to the retrospective character of the included studies, data extraction was often difficult. The included studies present heterogeneous data regarding tumour and treatment characteristics. None of the studies distinguished local recurrences from second primary tumours, and local recurrences were included independent of time interval or distance to the primary resection. Eight articles reported on additional treatment after the primary treatment (3-5, 28-31, 36). The influence of additional treatment on local recurrence risk was specified in one study (29). In nine studies, patients with a tumour-positive margin could be distinguished from the total group of patients with a tumour-free margin of <8 mm (3-5, 19, 28-30, 32, 36).

Table 1: Descriptive characteristics of the studies included in the meta-analysis

				Local recurrence risk				
Reference	**Design and inclusion period**	**No of partici-pants**	**Definition local recurrence**	**Tumour-positive margin**	**Tumour-free margin < 8 mm**	**Tumour-free margin ≥ 8 mm**	**Adjuvant treatment**	**Notes**
Baiocchi, 2015	Cohort study, (1980 – 2013)	205	Not specified	0	18/79	29/126	RT: 2 (2%) of 126 ≥ 8 mm and 8 (10%) of 79 < 8 mm	Influence of adjuvant therapy on local recurrence rate not further specified
Chan, 2007	Cohort study, (1984 – 2002)	90	Not specified	1/7	12/53	0/30	RT: 18 (20%) of 90 on the groins and/or perineum	Influence of adjuvant therapy on local recurrence rate not further specified
De Hullu, 2002	Cohort study, 15 years (1982 – 1997)	79	Histologically confirmed recurrence within 2 or 4 years after primary treatment	0/2	9/38	0/39	RT: 1 patient with a tumour positive margin	
Groenen, 2010	Cohort study, (2000 – 2005)	93	The period from the date of surgery till the clinically and histologically confirmed date of relapse	3/13	11/50	7/30	Re-excision: 13 (26%) of 50 < 8 mm and 7 (54%) of 13 tumour-positive RT: 11 (22%) of 50 < 8 mm and 2 (15%) of 13 tumour-positive	Influence of adjuvant therapy on local recurrence rate further specified. The number of local recurrence was comparable between the patients that did and did not receive adjuvant therapy
Heaps, 1990	Cohort study, (1957 – 1985)	135	Not specified	4/7	17/37	0/91	NS	Patients with a local recurrence more often had a stage 3 or 4 VSCC
Iacoponi, 2013	Cohort study, (2000 – 2010)	87	The appearance of tumour in a new location after a minimum disease-free period of 6 months	0/1	7/22	24/65	RT: 35 (40%) of 87	Influence of adjuvant therapy on local recurrence rate not further specified

Rouzier, 2002	Cohort study, (1978 – 1999)	215	Any tumour recurrence involving the skin and the subcutaneous tissues located around the vulvectomy scar or involving the skin bridge between the vulvectomy and groin dissection areas	NS	15/44	18/171	RT: 30 (68%) of 44 < 1 cm	Patients with a tumour-positive margin are included in the group of patients with a tumour-free margin < 1 cm 1 cm as cut-off value instead of 8 mm
Tantipalakorn, 2009	Cohort study, (1987 – 2005)	116	A recurrence within 2 cm of the primary tumour site, > 2 cm of the primary tumour site and skin bridge recurrence (in the dermis and the subcutaneous tissue between the groin and vulvar incisions)	0	8/24	17/92	NS	All patients had stage 1 or 2 VSCC Three types of recurrence described, but not specified for each patient group
Viswanathan, 2013	Cohort study, (1988 – 2009)	205	Recurrence free survival: the interval from diagnosis of primary disease to the date of first evidence of disease recurrence or progression or death from any cause.	9/20	44/116	9/69	RT: 11 (16%) of 69 ≥ 1 cm, 24 (21%) of 116 < 1 cm, 4 (20%) of 20 tumour-positive	1 cm as cut-off value instead of 8 mm Influence of adjuvant therapy on local recurrence rate not further specified
Woelber, 2011	Cohort study, (1998 – 2008)	102	Not specified	0	7/72	3/30	RT: 22 (31%) of 72 < 8 mm and 4 (13%) of 30 ≥ 8 mm	Influence of adjuvant therapy on local recurrence rate not further specified

NS: non-significant;
RT: radiotherapy;
VSCC: vulvar squamous cell carcinoma

Four studies (4, 19, 28, 31) found an increased risk of local recurrence for patients with a tumour-free margin <8 mm, with risk ratios ranging from 3.2 to 84.7. It should be emphasised, though, that in one of these studies (risk ratio, 3.2 [95% confidence interval {CI}: 1.8 – 5.9]), patients with a tumour-positive margin were included in the group of patients with a tumour-free margin of <8 mm (31). Six studies (3, 5, 29, 30, 32, 36) found no clearly increased risk of local recurrence when comparing <8 mm versus ≥ 8 mm.

Pooled random effects meta-analysis of these studies involving 1278 VSCC patients and 255 local recurrences showed a twofold increase in the risk of local recurrence for patients with a tumour-free margin <8 mm versus ≥ 8 mm (risk ratio 1.99 [95%CI 1.1 – 3.5], Figure 2). I^2 of the pooled analysis was 73%. After exclusion of the two studies that used 1 cm as cutoff instead of 8 mm, the risk ratio for local recurrence was 1.8 (95% CI 0.9–3.9) (Supplementary figure 1) (4, 31). After exclusion of the study that included patients with a positive margin in the <8 mm group, the pooled risk ratio was 1.88 (95% CI 0.99–3.5) (31).

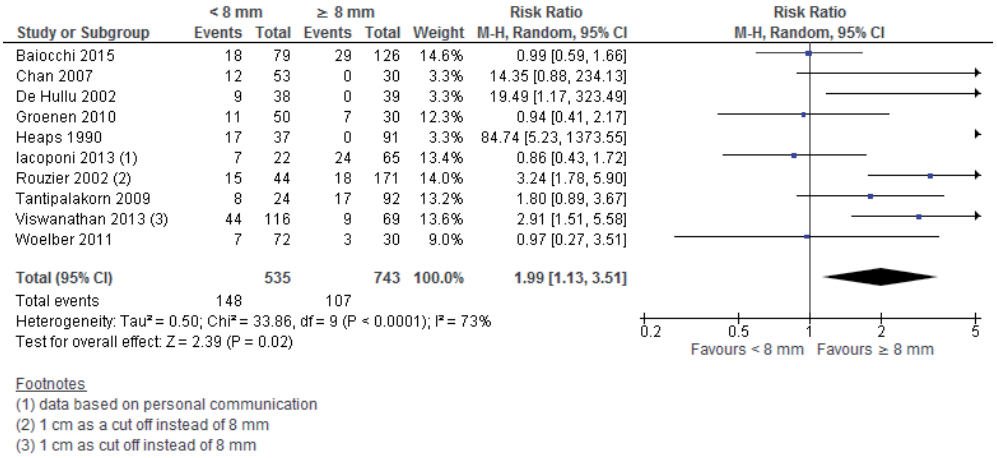


Figure 2: Meta-analysis. CI: confidence interval

Cohort study

Patient characteristics

Between January 2000 and December 2012, 192 patients underwent primary surgical treatment for VSCC at the Leiden University Medical Centre and 148 patients met the inclusion criteria for our study. The 44 patients that were excluded had a tumour with an infiltration depth of <1 mm or no residual tumour in the surgical specimen after excision biopsy at another hospital. Patient characteristics are described in table 2. Thirty patients (20%) had a tumour-positive margin, 92 (62%) had a tumour-free margin <8mm, and 26 (18%) had a tumour-free margin of ≥8 mm. The patient groups

Table 2: Patient characteristics (n=148)

Clinicopathological characteristics	**Tumour-positive margin (n=30)**	**Tumour-free margin < 8mm (n= 92)**	**Tumour-free margin ≥ 8mm (n= 26)**	**p-value**
Age (mean in years)	75	68	69	0.109
FIGO 2009				0.237
Stage I	12 (40.0%)	57 (62.0%)	18 (69.2%)	
Stage II	2 (6.7%)	2 (2.2%)	0 (0%)	
Stage III	15 (50.0%)	32 (34.8%)	8 (30.8%)	
Stage IV	1 (3.3%)	1 (1.1%)	0 (0%)	
Tumour size				< 0.001
Tumour size ≤ 40mm	13 (43.3%)	73 (79.3%)	23 (88.5%)	
Tumour size > 40mm	17 (56.7%)	19 (20.7%)	3 (11.5%)	
Depth of invasion				0.527
Depth of invasion ≤ 4mm	8 (26.7%)	37 (40.2%)	12 (46.2%)	
Depth of invasion > 4mm	22 (73.3%)	55 (59.8%)	14 (53.8%)	
LVSI				0.177
Yes	8 (26.7%)	16 (17.4%)	2 (7.7%)	
No	22 (73.3%)	76 (82.6%)	24 (92.3%)	
Primary treatment vulva				0.190
Radical local excision	12 (40.0%)	54 (58.7%)	13 (50.0%)	
Vulvectomy	18 (60.0%)	38 (41.3%)	13 (50.0%)	
Additional therapy				<0.001
Vulvar radiotherapy	20 (66.7%)[1]	22 (23.9%)	1 (3,8%)[3]	
Re-excision	7 (23.3%)[1]	15 (16.3%)	0 (0%)	
None	4 (13,3%)[2]	55 (59,8%)	25 (96,2%)	
Lymph node status				
Tumour-positive lymph nodes in the groin(s)	17 (56.7%)	33 (35.9%)	9 (34.6%)	0.108
Extracapsular spread	8 (26.7%)	13 (14.1%)	3 (11.5%)	0.210
Recurrence				
Local recurrence[4]	9 (30.0%)	9 (9.8%)	3 (11.5%)	0.010
Total recurrences[5]	12 (40.0%)	24 (26.1%)	6 (23.1%)	0.009
Median follow up time (months)	16	44	47	0.033

FIGO: Federation of Gynaecology and Obstetrics
LVSI: lymphovascular space invasion

[1] One patient with a tumour-positive histological margin received radiotherapy and reexcision as adjuvant treatment.

[2] Although indicated, four patients with a tumour-positive margin did not receive adjuvant therapy; one patient had metastasised disease and received palliative treatment only, one patient could not undergo radiotherapy because of severe comorbidity, one patient suffered from impaired wound healing and therefore an expectant management was and one patient died a few days postoperatively.

[3] This patient had an indication for postoperative radiotherapy on the inguinal region and simultaneously received radiotherapy on the vulva.

[4] Local recurrence: a histologically confirmed recurrence within 2 years after primary tumour on the ipsilateral side of the vulva.

[5] Total recurrences: all histologically confirmed recurrences on the vulva, irrespective of time and localisation.

were comparable for age, Federation of Gynaecology and Obstetrics (FIGO) stage, depth of invasion, primary treatment of the vulva, and lymph node status. Tumour size was larger in patients with a tumour-positive margin. Review of the tumour-free margins by an expert gynaecopathologist resulted in adjustment of the patient group in six cases (five patients initially had a tumour-free margin ≥8 mm according to the pathology report, but after revision had a tumour-free margin of <8 mm; another patient had a tumour-free margin <8 mm, but after revision it was ≥8 mm). Median follow-up time was 42 months (mean, 53.8 [range, 0–174] months).

Additional treatment was given to 26 of 30 patients (87%) with a tumour-positive margin (Figure 3). In the group with a tumour-free margins <8 mm, 37 of 92 patients (40%) received additional treatment (16% reexcision, 24% radiotherapy). Within this group, the mean tumour-free margin for patients who did and did not receive additional treatment was 3.1 mm (range, 0.31–7.85 mm) and 4.4 mm (range, 0.8–7.86), respectively.

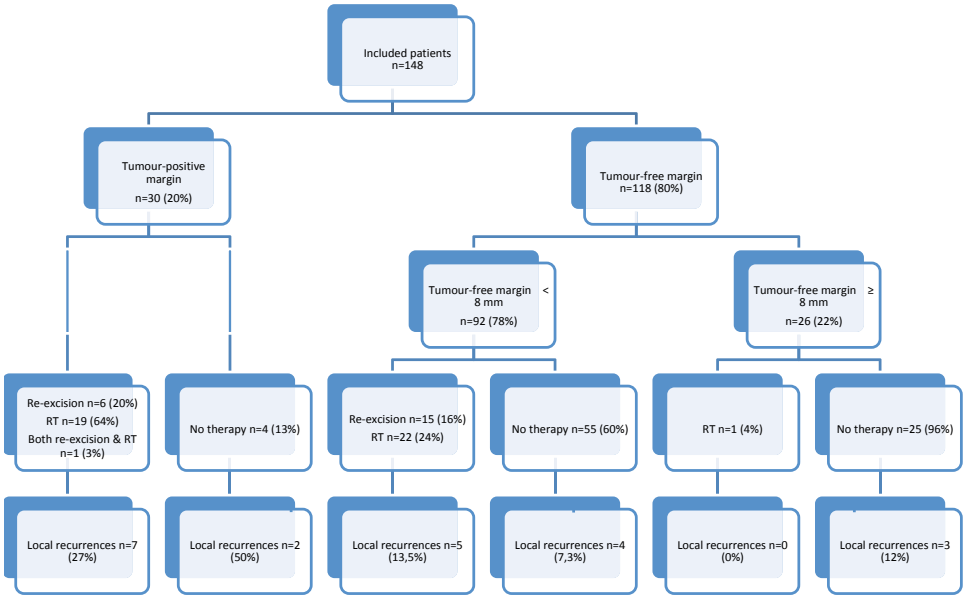


Figure 3: flowchart of margin distance, adjuvant treatment strategy and local recurrence rate

Risk of recurrence

Twenty-one of 148 patients (14%) developed local recurrences on the ipsilateral side of the vulva within 2 years after primary treatment. Another 21 patients developed a new tumour on the contralateral side of the vulva and/or more than 2 years after primary treatment, which were considered second primary tumours in this study (Table

2). In nine of these patients, the tumour developed on the ipsilateral side of the vulva. The competing risk analysis showed a cumulative incidence for local recurrence of 31% for patients with a tumour-positive margin, 10% for patients with a tumour-free margin of <8 mm, and 12% for patients with a tumour-free margin of ≥8 mm (p=0.01; Supplementary figure 2). There was no significant difference regarding local recurrence risk between the group of patients with a tumour-free margin of <8 mm versus ≥ 8 mm (hazard ratio [HR], 1.18 [95%CI: 0.32 – 4.35]).

Figure 3 displays the number of local recurrences in the different patient groups, taking additional treatment into account. Within the <8-mm group, there was no clear difference in local recurrence risk for patients who received additional treatment compared to patients who had no additional treatment (14% versus 7%, p=0.323). Of note, patients who received additional treatment more often had a higher FIGO stage, positive lymph nodes, and extracapsular growth of lymph node metastases, which are all known risk factors for local recurrence (data not shown) (20, 21). When analysing other tumour-free margins of 2, 4, and 6 mm, we found no differences in local recurrence risk (Table 3).

Table 3: local recurrence rate for other tumour-free margins

	2 mm	4 mm	6 mm
< tumour-free margin	2/19 (10.5%)	4/44 (9.1%)	7/74 (9.5%)
≥ tumour-free margin	10/99 (10.1%)	8/74 (10.8%)	5/44 (11.4%)
p-value	NS	NS	NS

NS: non-significant

Analysis of all "recurrences", irrespective of time and localisation on the vulva, showed no significant difference between the group of patients with a tumour-free margin of <8 mm and those with ≥8 mm (p=0.766) (data not shown).

Univariable and multivariable analysis

The results of the univariable and multivariable analyses for local recurrent disease of the vulva are shown in table 4. In univariable analysis, FIGO stage, positive lymph nodes, extracapsular growth, and the presence of a tumour-positive margin were associated with local recurrence. The only predictive factor for risk of local recurrence in the multivariable analysis was the presence of a tumour-positive margin versus a tumour-free margin of <8 mm (HR, 0.21 [95%CI 0.08–0.55]). A tumour-free margin of <8 mm did not clearly increase the risk of local recurrence compared to a tumour-free

margin of ≥8 mm in both univariable and multivariable analysis (HR, 1.18 [95% CI: 0.32–4.35] and HR, 1.09 [95% CI: 0.28–4.19], respectively). We performed a separate multivariable analysis on the influence of additional treatment on local recurrence risk in the group of patients with a tumour-free margin of <8 mm and corrected for FIGO stage, positive lymph nodes, extracapsular growth, and tumour-free margin distance. Patients who received additional treatment had a HR of 1.16 (95% CI: 0.23–5.84) for local recurrence compared to patients who did not (data not shown).

Table 4: Univariable and multivariable analysis for local recurrence

Predictors of local recurrence	Univariable analysis		Multivariable analysis	
	Hazard Ratio (CI)	p-value	Hazard Ratio (CI)	p-value
Age	1.02 (0.99 – 1.05)	0.281		
Tumour characteristics				
Tumour diameter ≤4 cm	1			
Tumour diameter >4 cm	1.43 (0.56 – 3.70)	0.456		
Tumour infiltration <4mm	1			
Tumour infiltration ≥4mm	1.48 (0.59 – 3.67)	0.396		
FIGO				
Stage 1&2	1		1	
Stage 3&4	2.73 (1.15 – 6.51)	**0.023**	1.67 (0.59 – 4.76)	0.339
Lymph node status				
Tumor negative	1		1	
Tumor positive	2.73 (1.15 – 6.51)	**0.023**	1.67 (0.59 – 4.76)	0.339
Extra capsular growth				
No	1		1	
Yes	3.20 (1.24 – 8.29)	**0.017**	2.53 (0.79 – 8.13)	0.120
Additional vulvar treatment				
No	1			
Yes	1.93 (0.81 – 4.59)	0.132	*	
HPV				
Negative	1			
Positive	0.24 (0.03 – 1.80)	0.240		
Margins				
< 8 mm versus positive margin	0.22 (0.09 – 0.55)	**0.001**	0.21 (0.08 – 0.55)	**0.001**
≥ 8 mm versus positive margin	0.25 (0.07 – 0.94)	**0.041**	0.29 (0.08 – 1.11)	0.070
< 8 mm versus ≥ 8 mm	1.18 (0.32 – 4.35)	0.808	1.09 (0.28 – 4.19)	0.903

CI: confidence interval
FIGO: Federation of Gynaecology and Obstetrics
HPV: human papillomavirus
*No multivariable analysis as in the group ≥8 mm only one patient was additionally treated

Discussion

Most guidelines recommend a tumour-free margin of ≥8 mm in the surgical treatment of VSCC (12, 13, 26), a recommendation that is mostly consensus based and supported by a lower level of evidence. To investigate whether tumour-free margins <8 mm are associated with an increased local recurrence risk in patients with primary VSCC, a meta-analysis was performed. This analysis showed a twofold increase in the local recurrence risk for patients with a tumour-free margin of <8 mm versus ≥8 mm. Nevertheless, there were substantial challenges regarding this meta-analysis. All included studies were retrospective, which made extraction of the necessary data difficult. Furthermore, it is imaginable that recurrences were missed due to the retrospective character of the studies, causing a reporting bias. Moreover, the included studies presented highly heterogeneous results. This might be partly explained by the different definitions of local recurrence used in the studies and missing data on additional treatment. Besides the meta-analysis, we performed a cohort analysis using a strict definition of local recurrence and considering the effect of additional treatment. In our cohort study, local recurrence risk within 2 years after primary surgery on the ipsilateral side of the vulva was 14%. We found no clear difference in local recurrence risk for patients with a tumour-free margin of <8 mm versus ≥ 8 mm. Importantly, a post hoc analysis of tumour-free margins of 2, 4, and 6 mm showed no difference in local recurrence risk. In a multivariable analysis, tumour-positive margins were the only independent risk factor for local recurrence.

In the meta-analysis, a total of 1278 VSCC patients from 10 studies were included. However, as mentioned above, statistical heterogeneity between the studies included was considerable (I^2=73%), and the results are therefore not easy to apply to individual patients. The local recurrence risk was 20%, which is consistent with the local recurrence risk found in other studies (20, 21). Definitions of local recurrence were different or not reported in the included studies (Table 1) (3, 5, 19). Other studies did not describe the distance to the primary tumour and/or the time span until a local recurrence (4, 28-32, 36). This can result in an overestimation of local recurrence risk because 'true local recurrences', as well as 'second primary tumours', are considered local recurrences. One study found that 14/52 (27%) 'local recurrences' were detected more than 2 years after primary treatment (28), and other studies showed that the maximum time to local recurrence could be as long as 166 months (4, 30, 36). It is unlikely that the size of the tumour-free margin has an influence on these 'late recurrences' or rather 'second primary tumours', which is also illustrated by the finding that remote site vulvar recurrences in general have a longer time to recurrence than primary site recurrences (31, 32). Indeed, in our cohort study we found that 21/42 (50%) newly developed tumours developed after more than 2 years or on the contralateral side of the vulva. Analysis of all "recurrences" in our cohort study, irrespective of time and localisation on the vulva, showed no significant

difference between patients with a tumour-free margin of <8 mm versus ≥8 mm (p=0.729). However, to definitely distinguish local recurrence from a second primary tumour, clonal or genetic relationship analysis should be performed (24).

Currently, there is very limited evidence on the effect of additional treatment (reexcision or adjuvant radiotherapy) with respect to the reduction of local recurrences after surgery in different tumour types. Importantly, randomised trials are lacking. A recent cohort study in 85 breast cancer patients with short tumour-free margins (≤ 2 mm) after breast-conserving surgery found a similar local recurrence risk for patients who underwent a reexcision (53%) versus those that did not (47%) (p=0.67) (37). To our knowledge, there are no studies on the impact of reexcision on local recurrence risk after primary treatment for VSCC. One cohort study including 34 VSCC patients with a tumour-free margin <8 mm investigated the influence of adjuvant radiotherapy and found a reduction in isolated local recurrence risk from 33% to 5% after adjuvant radiotherapy (33).

Missing data on additional treatment was a major limitation in the interpretation of the results of the meta-analysis, which hampered any conclusions on treatment effects. Only one study specified additional treatment in patients with a tumour-free margin <8 mm and found no difference in local recurrence risk (29). In our cohort study, 40% of patients in the <8 mm group received additional treatment. In these patients, the local recurrence risk was not different than that of patients who did not receive additional treatment. However, it should be considered that the patient group receiving additional treatment more often had a higher FIGO stage, positive lymph nodes, and extracapsular growth of lymph node metastases, which are all known prognostic factors that could influence the local recurrence risk (20, 21). Due to these limited data, it is not possible to make a final conclusion on the value of adjuvant treatment in patients with a tumour-free margin of < 8 mm.

In this meta-analysis and cohort study, we focused on 8 mm as a cutoff value for the tumour-free margin because this tumour-free margin is recommended in the Dutch and US guidelines (12, 13, 26). A post hoc analysis in our cohort study for tumour-free margins of 2, 4, and 6 mm showed no difference in local recurrence risk. There are few other studies that examined tumour-free margins other than 8 mm in the surgical treatment of VSCC (3 and 5 mm). In two studies, no difference in local recurrence risk was found for a tumour-free margin of 3 or 5 mm (5, 38). In contrast, Viswanathan et al. described a significantly reduced local recurrence risk for tumour-free margins ≥5 mm (HR 0.53 [95% CI 0.3–0.9]) (4). Two other studies defined a positive margin as <3 mm and found an increased local recurrence risk for patients with a 'tumour-positive' margin. However, these studies did not describe whether patients with tumour-positive margins were also included in the <3 mm patient group (39, 40).

In summary, currently, there is no firm evidence on the optimal length of the tumour-free margin in the treatment of VSCC. Due to the low incidence of vulvar cancer, there are no large prospective studies concerning this important clinical issue. This work provides important data to question the commonly used 8 mm margin as a prognosticator for local recurrence. More research is needed to address the question of whether additional treatment improves the prognosis in patients with a tumour-free margin smaller than 8 mm and what the best cutoff for the tumour-free margin would be.

Conflict of interest statement
None declared

Appendix A

Search string for meta-analysis:
("Vulvar Neoplasms"[Mesh] OR "vulva carcinoma"[all fields] OR "vulvar carcinoma"[all fields] OR "vulva carcinomas"[all fields] OR "vulvar carcinomas"[all fields] OR "Vulvar Neoplasm"[all fields] OR "Vulva Neoplasms"[all fields] OR "Vulvar Neoplasms"[all fields] OR "Cancer of Vulva"[all fields] OR "Vulva Cancers"[all fields] OR "Cancer of the Vulva"[all fields] OR "Vulva Cancer"[all fields] OR "Vulvar Cancer"[all fields] OR "Vulvar Cancers"[all fields] OR "vulval carcinoma"[all fields] OR "vulval carcinomas"[all fields] OR "Vulval Neoplasm"[all fields] OR "Vulval Neoplasms"[all fields] OR "Vulval Cancers"[all fields] OR "Vulval Cancer"[all fields] OR "vulva neoplasia"[all fields] OR "vulvar neoplasia"[all fields] OR "vulval neoplasia"[all fields]) AND ("surgical margin"[all fields] OR "histological margin"[all fields] OR "surgical margins"[all fields] OR "histological margins"[all fields] OR "surgical excision margin"[all fields] OR "surgical excision margins"[all fields] OR "clinical margin"[all fields] OR "clinical margins"[all fields] OR "margin assessment"[all fields] OR "histopathologic margin"[all fields] OR "excision margin"[all fields] OR "tumor margin"[all fields] OR "tumour margin"[all fields] OR "histopathologic margins"[all fields] OR "excision margins"[all fields] OR "tumor margins"[all fields] OR "tumour margins"[all fields] OR "margin"[all fields] OR "margins"[all fields])

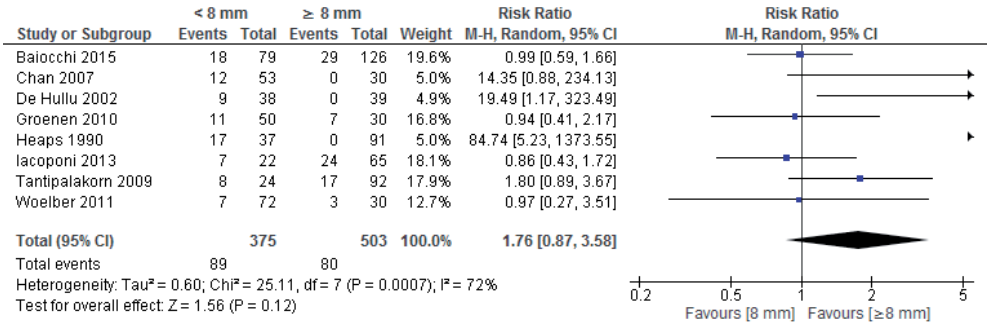
References

1. Hacker NF, van d, Velden. Conservative management of early vulvar cancer. Cancer. 1993;71(4 Suppl):1673-7.
2. Alicandri-Ciufelli M, Bonali M, Piccinini A, Marra L, Ghidini A, Cunsolo EM, et al. Surgical margins in head and neck squamous cell carcinoma: what is 'close'? EurArchOtorhinolaryngol. 2013;270(10):2603-9.
3. Chan JK, Sugiyama V, Pham H, Gu M, Rutgers J, Osann K, et al. Margin distance and other clinico-pathologic prognostic factors in vulvar carcinoma: a multivariate analysis. Gynecol Oncol. 2007;104(3):636-41.
4. Viswanathan AN, Pinto AP, Schultz D, Berkowitz R, Crum CP. Relationship of margin status and radiation dose to recurrence in post-operative vulvar carcinoma. Gynecol Oncol. 2013;130(3):545-9.
5. Woelber L, Choschzick M, Eulenburg C, Hager M, Jaenicke F, Gieseking F, et al. Prognostic value of pathological resection margin distance in squamous cell cancer of the vulva. Ann Surg Oncol. 2011;18(13):3811-8.
6. Anderson CR, Sisson K, Moncrieff M. A meta-analysis of margin size and local recurrence in oral squamous cell carcinoma. Oral Oncol. 2015;51(5):464-9.
7. Luini A, Rososchansky J, Gatti G, Zurrida S, Caldarella P, Viale G, et al. The surgical margin status after breast-conserving surgery: discussion of an open issue. Breast Cancer ResTreat. 2009;113(2):397-402.
8. Gunia S, Koch S, Jain A, May M. Does the width of the surgical margin of safety or premalignant dermatoses at the negative surgical margin affect outcome in surgically treated penile cancer? JClinPathol. 2014;67(3):268-71.
9. Byerly S, Chopra S, Nassif NA, Chen P, Sener SF, Eisenberg BL, et al. The role of margins in extremity soft tissue sarcoma. JSurgOncol. 2015.
10. Dickinson IC, Whitwell DJ, Battistuta D, Thompson B, Strobel N, Duggal A, et al. Surgical margin and its influence on survival in soft tissue sarcoma. ANZJSurg. 2006;76(3):104-9.
11. Motley R, Kersey P, Lawrence C. Multiprofessional guidelines for the management of the patient with primary cutaneous squamous cell carcinoma. BrJDermatol. 2002;146(1):18-25.
12. National Dutch Guideline Gynaecologic Tumors; Vulvar Carcinoma. http://www.oncoline.nl ed2015.
13. National Cancer Institute. Vulvar Cancer Treatment. Vulvar Cancer Treatment. http://www.cancer.gov ed2015.
14. Tirelli G, Zacchigna S, Biasotto M, Piovesana M. Open questions and novel concepts in oral cancer surgery. EurArchOtorhinolaryngol. 2015.
15. Dik EA, Willems SM, Ipenburg NA, Adriaansens SO, Rosenberg AJ, van Es RJ. Resection of early oral squamous cell carcinoma with positive or close margins: relevance of adjuvant treatment in relation to local recurrence: margins of 3 mm as safe as 5 mm. Oral Oncol. 2014;50(6):611-5.
16. Wood WC. Close/positive margins after breast-conserving therapy: additional resection or no resection? Breast. 2013;22 Suppl 2:S115-S7.
17. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.

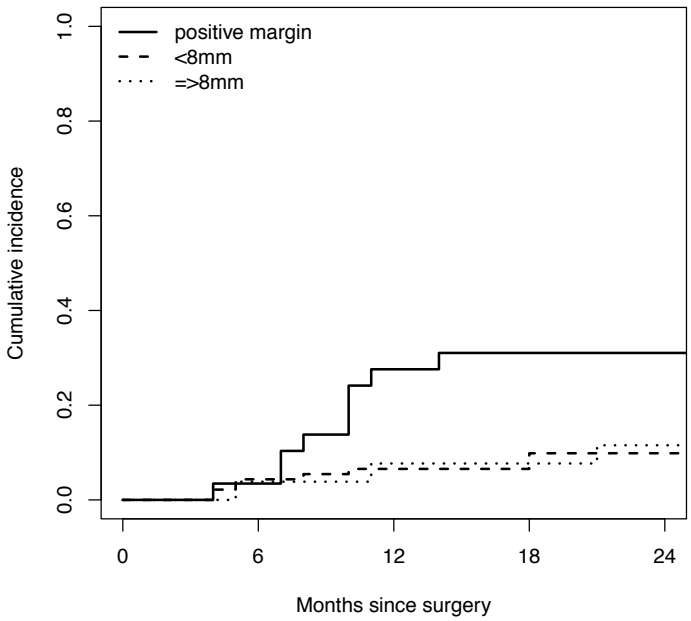
18. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. Histopathology. 2013;62(1):161-75.
19. Heaps JM, Fu YS, Montz FJ, Hacker NF, Berek JS. Surgical-pathologic variables predictive of local recurrence in squamous cell carcinoma of the vulva. Gynecol Oncol. 1990;38(3):309-14.
20. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
21. Coulter J, Gleeson N. Local and regional recurrence of vulval cancer: management dilemmas. Best Pract Res Clin Obstet Gynaecol. 2003;17(4):663-81.
22. Te Grootenhuis NC, van der Zee AG, van Doorn HC, van d, V, Vergote I, Zanagnolo V, et al. Sentinel nodes in vulvar cancer: Long-term follow-up of the GROningen INternational Study on Sentinel nodes in Vulvar cancer (GROINSS-V) I. GynecolOncol. 2016;140(1):8-14.
23. Torezan LA, Festa-Neto C. Cutaneous field cancerization: clinical, histopathological and therapeutic aspects. AnBrasDermatol. 2013;88(5):775-86.
24. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res. 2003;63(8):1727-30.
25. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. Crit RevOncolHematol. 2008;68(2):131-56.
26. National Comprehensive Cancer Network guideline on vulvar cancer. https://www.nccn.org/store/login/login.aspx?ReturnURL=http://www.nccn.org/professionals/physician_gls/pdf/vulvar.pdf ed2016.
27. RCOG guideline vulval carcinoma. https://www.rcog.org.uk/globalassets/documents/guidelines/vulvalcancerguideline.pdf ed2015.
28. de Hullu JA, Hollema H, Lolkema S, Boezen M, Boonstra H, Burger MP, et al. Vulvar carcinoma. The price of less radical surgery. Cancer. 2002;95(11):2331-8.
29. Groenen SM, Timmers PJ, Burger CW. Recurrence rate in vulvar carcinoma in relation to pathological margin distance. Int J Gynecol Cancer. 2010;20(5):869-73.
30. Iacoponi S, Zapardiel I, Diestro MD, Hernandez A, De SJ. Prognostic factors associated with local recurrence in squamous cell carcinoma of the vulva. J Gynecol Oncol. 2013;24(3):242-8.
31. Rouzier R, Haddad B, Plantier F, Dubois P, Pelisse M, Paniel BJ. Local relapse in patients treated for squamous cell vulvar carcinoma: incidence and prognostic value. Obstet Gynecol. 2002;100(6):1159-67.
32. Tantipalakorn C, Robertson G, Marsden DE, Gebski V, Hacker NF. Outcome and patterns of recurrence for International Federation of Gynecology and Obstetrics (FIGO) stages I and II squamous cell vulvar cancer. ObstetGynecol. 2009;113(4):895-901.
33. Faul CM, Mirmow D, Huang Q, Gerszten K, Day R, Jones MW. Adjuvant radiation for vulvar carcinoma: improved local control. Int J RadiatOncol BiolPhys. 1997;38(2):381-9.
34. Viswanathan M AM, Berkman ND, Chang S, Hartling L, McPheeters LM, Santaguida PL, Shamliyan T, Singh K, Tsertsvadze A, Treadwell JR. Assessing the Risk of Bias of Individual Studies in Systematic Reviews of Health Care Interventions. March 2012.

35. Gadducci A, Cionini L, Romanini A, Fanucchi A, Genazzani AR. Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer. Crit RevOncolHematol. 2006;60(3):227-41.
36. Baiocchi G, Mantoan H, de Brot L, Badiglian-Filho L, Kumagai LY, Faloppa CC, et al. How important is the pathological margin distance in vulvar cancer? Eur J Surg Oncol. 2015;41(12):1653-8.
37. Biglia N, Ponzone R, Bounous VE, Mariani LL, Maggiorotto F, Benevelli C, et al. Role of re-excision for positive and close resection margins in patients treated with breast-conserving surgery. Breast. 2014;23(6):870-5.
38. Woelber L, Mahner S, Voelker K, Eulenburg CZ, Gieseking F, Choschzick M, et al. Clinicopathological prognostic factors and patterns of recurrence in vulvar cancer. Anticancer Res. 2009;29(2):545-52.
39. Preti M, Ronco G, Ghiringhello B, Micheletti L. Recurrent squamous cell carcinoma of the vulva: clinicopathologic determinants identifying low risk patients. Cancer. 2000;88(8):1869-76.
40. Rouzier R, Preti M, Haddad B, Martin M, Micheletti L, Paniel BJ. Development and validation of a nomogram for predicting outcome of patients with vulvar cancer. Obstet Gynecol. 2006;107(3):672-7.

Supplementary data



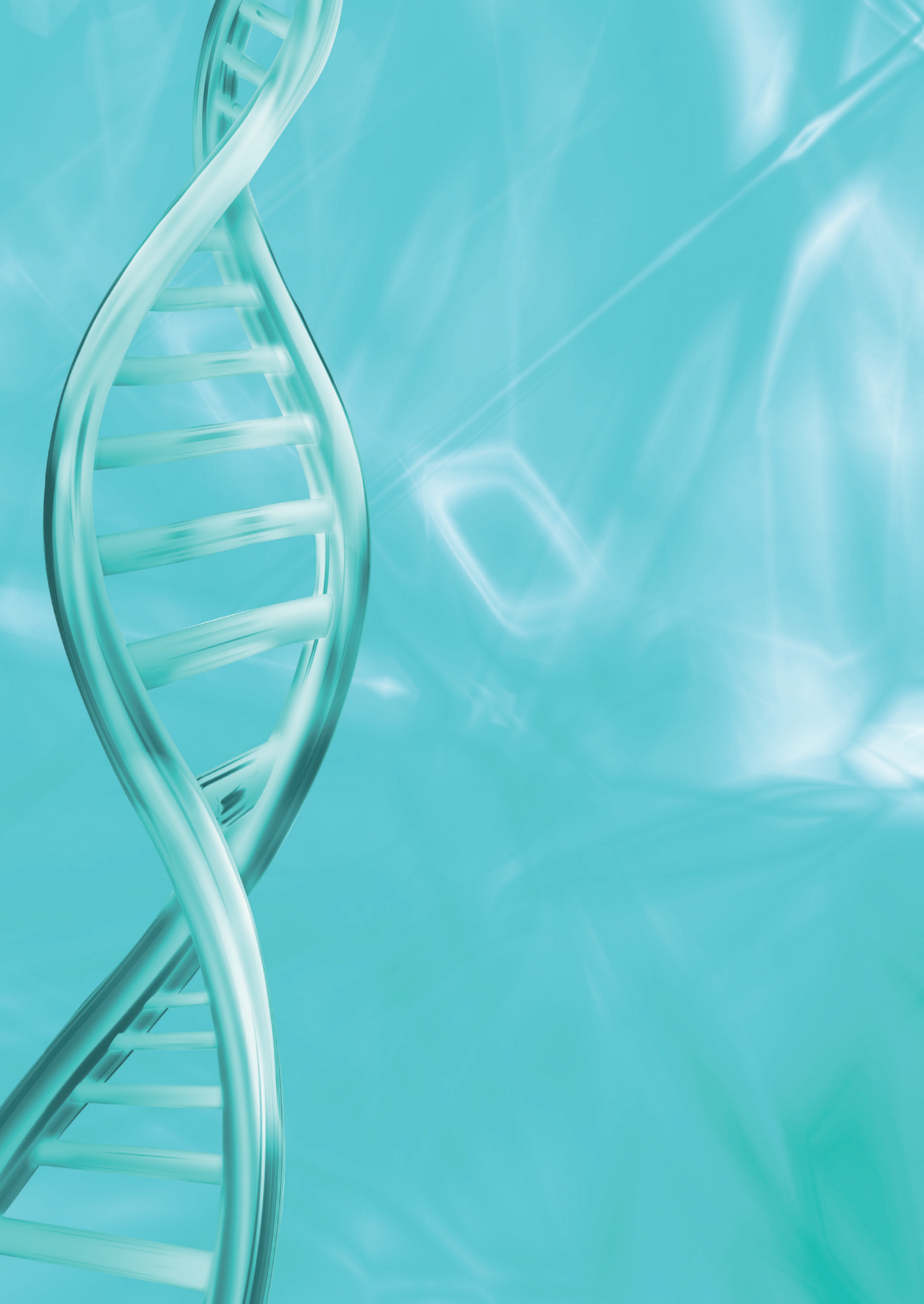
Supplementary figure 1: Results meta-analysis after exclusion of two studies with 1 cm as a cutoff



Supplementary figure 2: Cumulative incidence for local recurrence

Supplementary table 1: Risk of bias analysis

Study	Selection bias (consecutive patients or a random sample)	Performance bias (Unequal co-interventions during follow-up?)	Attrition bias (loss to follow-up related to outcome?)	Detection bias (unequal length of follow-up?)	Reporting bias (no histological confirmation)
Baiocchi, 2015	Not described	Not described	Not described	Not described	Not described
Chan, 2007	No bias	Not described	Not described	Not described	Not described
De Hullu, 2002	No bias	No bias	No bias	Not described	No bias
Groenen, 2010	No bias	No bias	Not described	Not described	No bias
Heaps, 1990	No bias	Not described	Not described	Not described	Not described
Iacoponi, 2013	Not described	No bias	Not described	Not described	No bias
Rouzier, 2002	No bias	Not described	Not described	Not described	No bias
Tantipalakorn, 2009	Not described	No bias	Not described	Not described	Not described
Viswanathan, 2013	No bias	Not described	Not described	Not described	No bias
Woelber, 2011	No bias	Not described	Not described	Not described	Not described



CHAPTER 3

Groin surgery and risk of recurrence in lymph node positive patients with vulvar squamous cell carcinoma

Linda S. Nooij
Paulien J. Ongkiehong
Erik W. van Zwet
Tjalling Bosse
Carien L. Creutzberg
Katja N. Gaarenstroom
Mariette I.E. van Poelgeest

Gynecologic Oncology 2015;139:458-464

Abstract

Objectives: Treatment of groin metastasis in vulvar squamous cell carcinoma (VSCC) patients consists of surgery, often combined with (chemo)radiotherapy, and is associated with significant morbidity. Our aim was to compare the risk of groin recurrence and morbidity in patients with lymph node positive VSCC after standard full inguinofemoral lymphadenectomy (IFL) versus less radical debulking of clinically involved lymph nodes or removal of sentinel nodes only followed by radiotherapy.

Methods: A retrospective cohort study of 68 patients with primary VSCC and proven lymph node metastasis to the groin(s) was conducted. Patients were divided into three subgroups by type of initial groin surgery (84 groins): sentinel node (SN), IFL, and debulking of clinically involved nodes. Most patients (82%) received adjuvant radiotherapy. Overall survival was analyzed using time dependent cox regression. Analysis of morbidity and groin recurrence-free time was performed per groin with the generalized estimating equation model and Kaplan Meier method.

Results: There was no significant difference in the risk of developing a groin recurrence (SN 25%, debulking 16%, IFL 13%, p=0.495). Despite the fact that more patients received radiotherapy after debulking (90% vs 67%), the complication rate was significantly lower (p=0.003) compared to IFL, especially regarding lymphocysts and lymphedema (p=0.032 and p=0.002 respectively).

Conclusions: The risk of groin recurrence was similar in all treatment groups. Debulking of clinically involved lymph nodes was related to a significant lower risk of complications compared to IFL. These findings support that the preferred treatment of patients with clinically involved lymph nodes is debulking followed by radiotherapy.

Introduction

Vulvar cancer is a rare disease, representing about 4% of all female genital cancers. The majority of these tumors (90%) are vulvar squamous cell carcinomas (VSCC)(1). Most patients present with early stage disease, which has an excellent 5-year survival rate of up to 90% (2, 3). The presence of groin metastases is the most significant negative predictor for survival (2, 4, 5) with reported 5-year survival rates ranging from 0 to 51% (3, 6, 7). In VSCC patients with early stage disease the incidence of groin metastases is 20-30%, whereas in patients with advanced disease, the incidence can be as high as 60% (2, 3, 8, 9). Patients with groin metastases have an increased risk of groin recurrence (2, 8-11), which is fatal in almost all patients (12-14).

The cornerstone of treatment for vulvar cancer is surgery(1, 5). Surgery for all VSCC stages used to be extensive before 1980, consisting of en-bloc radical vulvectomy and bilateral dissection of the groins and enlarged pelvic nodes, leading to a high risk of morbidity, such as wound infections, wound breakdown, lymphocysts and lymphedema. Last decades, treatment consisted of local radical excision with separate groin incisions, with still high morbidity rates (9, 15-20). For patients with a unifocal tumor of 4 cm or less, without suspicious groin nodes on ultrasonography or CT-scan, the sentinel node (SN) procedure has proven to be a safe treatment, with reported groin recurrence rates of 2,3 – 3% (8, 9, 12, 21). These changes in surgical approach have resulted in a significant decrease in morbidity without causing an increase in mortality rate or recurrence risk (8, 15, 22). However, patients with multifocal disease, a tumor size larger than 4 cm, or clinically involved lymph nodes of the groin should receive radical treatment of the groin, consisting of either debulking of enlarged lymph nodes with postoperative radiotherapy, or inguinofemoral lymphadenectomy (IFL) (8, 21). After IFL, postoperative radiotherapy is advised for patients with two or more positive groin nodes or extracapsular spread of groin nodal involvement (1, 23).

Treatment of groin metastases of patients with VSCC remains a major challenge, because affected patients are mainly women over 60 years of age and have a high risk of developing complications after surgery (24-26). Extensive groin surgery as well as radiotherapy, and in particular the combination of these treatments, are associated with significant morbidity (5, 9, 16, 17, 20, 27-29). At present, it remains uncertain which surgical approach for the treatment of groin metastases has the best overall outcome with both lowest risk of groin recurrence and of complications. In 2007 Hyde et al. performed a retrospective study on a series of 40 VSCC patients with lymph node metastases to the groin who underwent either lymph node debulking or IFL followed by radiotherapy. In this study surgical treatment was performed in three different clinics in the Netherlands and in Australia. The results showed no difference in overall survival when groin surgery was followed by inguinal and pelvic radiation (30).

Our study aimed to investigate the risk of groin recurrences and overall survival in relation to surgical treatment in a larger group of patients with VSCC and cytologically or histologically proven metastases to the groin treated within one center. We investigated three different surgical approaches in case of proven metastases to the groin in patients with VSCC: SN procedure; debulking of clinically involved lymph nodes; or IFL. Additionally, we investigated the differences in morbidity between the different surgical groin treatment approaches.

Methods

Patients and treatment

A single-institution retrospective study was performed. Clinical and histopathological data of patients with newly diagnosed VSCC who were referred to the Leiden University Medical Center between January 2000 and December 2012 were collected. Only patients with cytologically or histologically proven lymph node metastases to the groin were included. Data collection was carried out according to the guidelines of the Ethics Committee of the Leiden University Medical Center.

Local vulvar cancer treatment consisted of radical vulvectomy, wide local excision or primary chemoradiation. Groin surgery consisted of either IFL, debulking of clinically involved groin nodes, SN procedure, or a combination of these treatment modalities to both groins. During the study period surgery was performed by the same four experienced gynecologic oncologists. IFL is defined as removal of all lymph node bearing fatty tissue between the inguinal ligament, the sartorius muscle and the adductor longus muscle and dissection of the femoral lymph nodes located in the fossa ovalis medial to the femoral vein (16, 30-32). Debulking is defined as selectively removing clinically involved or cytological/histological proven positive and enlarged groin nodes (30). The sentinel node procedure was performed using the radioactive tracer ^{99m}Tc-labelled colloid and blue dye as reported previously according to the GROINSS-V protocol (8, 33). From 2000 until 2010 we performed a debulking of clinically involved nodes generally followed by radiotherapy when metastases were detected pre- or intra-operatively by ultrasonography or frozen section or when the SN was tumor positive. An IFL was performed in patients with tumors larger than 4 cm when there were no clinically involved lymph nodes. From July 2010 onwards, patients with a macrometastasis of >2 mm in the SN underwent IFL, because this was reported to be a risk factor for groin recurrence (19). Patients with a micrometastasis < 2 mm in the SN without enlarged lymph nodes on ultrasound or CT scan were treated with additional radiotherapy, as well as patients with more than one groin metastasis and/or extracapsular spread at IFL. Patients with locally advanced disease were treated with concurrent chemoradiation as primary treatment after IFL or debulking of clinically involved nodes. Radiotherapy was

administrated to the inguinal and external iliacal regions, and in case of close margins also to the vulva, to a total dose of 46-50.5 Gy in 23-28 daily fractions of 1.8-2 Gy, 5 times a week, with a boost dose to 56-60 Gy to the involved groin in case of extracapsular extension. If primary chemoradiation was used, radiotherapy was combined with either oral capecitabine 825 mg/m2 on days 1-14 every 3 weeks (within a national phase 2 study) or with 5-fluorouracil (5FU) 1000 mg/m2 i.v 4x24 hr continuous infusion in weeks 1 and 5 of radiotherapy and mitomycin-C 10 mg/m2 i.v on day 1 of 5FU administration (unless clinically contraindicated). Patients were followed at 2-3 month intervals in the first two years after treatment, 4 to 6-monthly in the third and fourth year and annually thereafter.

Tumor characteristics that were analysed were tumor location, size, depth of infiltration and lymphovascular space invasion (LVSI). LVSI was considered positive when cancer cells were present within endothelium-lined spaces on regular hematoxylin and eosin stained slides. Furthermore, the total number of removed lymph nodes, the number of tumor positive lymph nodes, the size of groin metastases and intra- or extranodal growth were documented. In case of missing histopathological data, pathological slides were reviewed by a gynaecological pathologist (TB).

To study treatment-related morbidity, overall complication rate, the presence of lymphocysts, lymphedema, wound dehiscence, wound infection and wound hematoma were documented. These data were collected retrospectively from the patient charts. Complications were defined according to an earlier study performed in our clinic regarding complications after IFL procedure (16). Lymphocysts were documented if greater than 4 cm in diameter and generally confirmed by cytology and/or ultrasound. Lymphedema was defined when clinically relevant and/or if lymphedema treatment using manual compression therapy and compression stockings were required from three months after surgery onwards. Wound dehiscence was defined as disruption of the groin wound over more than one-third of the length of the incision. A wound infection was noted if a purulent exudate was present and/or if a patient had a positive wound culture with erythema, edema and localized pain requiring antibiotics (16).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 20. Clinical and histopathological characteristics of all patients were analysed with the with the Fisher exact test and one way ANOVA. Groin recurrence-free time was defined as the time from date of primary surgery until the date of histologically proven groin recurrence or date of last follow up or death. Overall survival is defined as the time from surgery until date of death, irrespective of the cause, with censoring at date of last follow up. Groin recurrence-free time was analysed per groin and estimated with the Kaplan-Meier method. Groins of the

patients were subdivided into three groups regarding the analysis of surgical treatment to the groin and groin recurrence: 1) SN procedure only, 2) lymph node debulking, 3) IFL. If a SN procedure was followed by either a debulking or IFL, the final surgical treatment was leading. Overall survival was analysed for the whole patient group, subdivided into patients who did and patients who did not develop a recurrence in the groin(s). Analysis of overall survival was done with cox regression, using a time dependent covariate to take the time to develop a recurrence into account.

Because some patients received different groin treatment modalities to both groins, a per groin analysis for the statistical analyses of the groin characteristics was performed. To take the possible correlation of both groins in one patient into account, analyses were done using the generalized estimating equations model. In this model we corrected for age.

A univariate and multivariate analysis was performed using the cox regression model in order to determine risk factors for groin recurrence. Clinicopathological variables that were considered in the analysis are: primary surgical treatment of the groin, age, FIGO stage, tumor size, LVSI, intact or extracapsular nodal growth of the groin metastasis, depth of infiltration of the primary tumor, the number of removed lymph nodes during groin surgery, the number of tumor positive lymph nodes, the size of the groin metastases and adjuvant groin treatment after initial surgery (i.e. radiotherapy). These variables are regarded as known risk factors influencing prognosis (10, 34-36). In the multivariate analysis we analysed primary groin treatment and all prognostic variables with a p-value of <0.1.

A p-value of <0.05 was considered to be statistically significant.

Results

Patients and treatments

From January 2000 to December 2012, 289 patients were treated for primary vulvar cancer at Leiden University Medical Center, of whom 232 (80%) presented with VSCC. Seventy-two (31%) of these 232 patients had histological proven metastases to the groin nodes, either diagnosed before or after surgery by cytological or histological examination. Of these 72 patients, one patient refused initial surgical treatment of groin metastasis and 3 patients received primary radiotherapy to the groins. These four patients were excluded from the analysis. Subsequently, the study group consisted of 68 patients with VSCC and cytologically or histologically proven lymph node metastases to the groin who underwent surgery of the groin as their initial treatment.

Table 1: Clinical and histopathological characteristics of 68 lymph node positive VSCC patients

		N (Range)	%
Mean age		70,8 (35-94)	
FIGO stage (2009)	III	54	79,4%
	IV	14	20,6%
Location vulva tumour	Midline	15	22%
	Unilateral	43	63,3%
	Bilateral	6	8,8%
	Multifocal	3	4,4%
	Not specified	1	1,5%
Size vulva tumour	≤ 2 cm	15	22,1%
	2 – 4 cm	27	39,7%
	≥ 4 cm	26	38,2%
Depth of infiltration vulva tumour	1-4 mm	21	30,9%
	≥ 4 mm	47	69,1%
Lymph vascular space invasion (LVSI)	No	45	66,2%
	Yes	19	27,9%
	Unknown	4	5,9%
Primary treatment vulva tumor	Wide local excision	53	77,9%
	Radical vulvectomy	12	17,7%
	Chemoradiation	3	4,4%
Primary groin treatment	SN unilateral	7	10,3%
	SN bilateral	5	7,4%
	Debulking unilateral	11	16,2%
	Debulking bilateral	7	10,3%
	IFL unilateral	2	2,9%
	IFL bilateral	18	26,5%
	Combination in both groins		
	IFL and debulking	13	19,1%
	IFL and SN	3	4,4%
	Debulking and SN	2	2,9%
Adjuvant treatment groin metastasis	None	12	17,6
	Radiotherapy	51	75%
	Chemoradiation	5	7,4%
Location groin metastasis	Unilateral	52	76,5%
	Bilateral	16	23,5%
Size of groin metastasis	Isolated tumour cells	7	10,3%
	≤2 mm	8	11,8%
	>2 mm	53	77,9%
Nodal growth groin metastasis	Intact	37	54,4%
	Extra capsular	30	44,1%
	Not assessable	1	1,5%
Patients with groin recurrence		14	20,6%
Mean time until groin recurrence (months)		14,4 (2-39)	
Current patient status at end of follow up or last visit	Alive, recurrence free	17	25%
	Alive, recurrent disease	2	2,9%
	Death because of tumour	34	50%
	Death, other cause	14	20,6%
	Death, unknown cause	1	1,5%

VSCC: vulvar squamous cell carcinoma

Clinical and histopathological characteristics of the study group are shown in table 1. Mean age was 70.8 years. Initial surgical treatment of the groins consisted of a SN procedure only in 12 patients; debulking of clinically involved or enlarged lymph nodes in 18 patients; IFL in 20 patients; and a combination of surgical procedures to both groins in 18 patients. Sixty-five of the 68 patients (96%) underwent primary surgical treatment of the vulva tumor. Three patients with locally advanced disease were treated with primary chemoradiation on the vulva in combination with lymph node debulking of one groin. Fifty-one (75%) of the 68 patients received adjuvant radiotherapy to the groins and 5 patients (7%) underwent chemoradiation. In all patients who received adjuvant (chemo)radiation a CT-scan was performed for planning of the radiotherapy. In the rare cases that borderline residual nodes were seen on a radiotherapy planning CT scan, a repeat ultrasound with cytology was done that was negative in all cases. In 9 of 12 patients who did not receive adjuvant therapy, pre-operative imaging had been performed to exclude enlarged lymph nodes consisting of an ultrasound in 7 patients, a CT-scan in 1 patient and MRI-scan in 1 patient. Adjuvant treatment was started as soon as possible after surgery. Forty-three patients (77%) started with radiotherapy within six weeks after surgery. For thirteen patients (23%) radiotherapy was started later than six weeks after treatment. Eleven of these patients started adjuvant treatment within eight weeks after surgery. The reason for delay for the other two patients was that one patient refused adjuvant radiotherapy at first and the other patient had prolonged wound recovery. For three patients a break during radiation therapy was needed: one patient had a break of two days on her own request; one had a break of one week due to extensive moist desquamation, after which the radiotherapy was completed according to the normal schedule. The third patient discontinued radiotherapy shortly after the start due to a rapid deterioration of her condition because of metastatic disease. Twelve (18%) of the 68 patients did not receive adjuvant radiotherapy, despite the presence of lymph node metastases: for 8 patients this was not considered necessary because of a single metastasis to the groin node without extracapsular growth. Four of these patients had a micrometastasis. The four patients who had a metastasis of >2 mm after IFL were all treated before the GROINSS-V-II amendment in 2010 and therefore did not receive radiotherapy. One patient died shortly after primary surgical treatment and 2 patients refused adjuvant radiotherapy. These three patients underwent a SN procedure. Finally, 1 out of these 12 patients underwent debulking to one groin and IFL to the other groin. In the latter patient, a total of 20 lymph nodes in the groin were excised, all of which were tumor positive with extensive extranodal growth. Additional radiological imaging showed pulmonary metastasis. Therefore it was decided to start palliative treatment. Mean follow up time of all patients was 33,4 months (range 0-146 months, median follow up time 20,5 months).

Clinical outcome: overall survival and groin recurrences

Forty-nine (72%) of 68 patients died, 34 of whom due to recurrent or progressive disease. For 1 patient in the IFL group the cause of death was unknown. Fourteen (21%) of 68 patients developed a groin recurrence, with a mean time until recurrence of 14 months (range 2-39 months). Three out of these fourteen patients (21,4%) had an ipsilateral vulvar recurrence at the same time of the groin recurrence.

Twelve (86%) out of 14 patients with a groin recurrence died. Mean and median overall survival for patients with a groin recurrence was 20 and 18 months, respectively (range 6-43 months). Eleven patients with a groin recurrence (79%) died because of the disease. In the group of patients without a groin recurrence 37 patients (69%) died, 23 (42,6%) due to disease. Mean and median overall survival in this group was 37 and 23 months (range 0-146 months). The hazard ratio (HR) of dying was nine (8,995) times higher for patients who developed a groin recurrence compared to patients who did not develop a groin recurrence ($p<0.001$).

Per-groin analysis

In 68 patients, 116 groins were treated surgically. Eighty-four of these groins had lymph node metastases and were eligible for analysis (Table 2). In 16 groins (19%) a SN procedure was performed, 38 groins (45%) were treated with debulking surgery, and thirty groins (36%) with IFL. The size of the primary vulva tumor was significantly larger in the debulking group compared to the other treatment groups ($p=0.005$). Pre-operative suspicious lymph nodes were present in 34 patients (89,5%) of the debulking group versus 17 patients (56,7%) in the IFL group . This difference remained significant when comparing these last two treatment groups ($p<0.001$). As expected, the number of removed lymph nodes was significantly higher in the IFL group compared to the other treatment groups ($p<0.001$). The number of tumor-positive lymph nodes was not significantly different between the treatment groups ($p=0.140$). The number of groin metastases with extra capsular growth was significantly higher in the debulking group compared to the SN procedure only and IFL group (68% versus 19% and 47% respectively, $p=0.002$). Macro-metastases (>2 mm) were found significantly more often in the debulking (95%) and IFL groups (93%) compared to the SN group (25%) ($p<0.001$). More patients in the debulking group (90%) received adjuvant radiotherapy compared to the SN (69%) and IFL (67%) groups ($p=0.013$).

Table 2: Groin characteristics, complications and recurrences among 84 surgically treated groins in 68 patients

Variables		SN procedure N=16	Debulking N=38	IFL N=30	P value
Size vulva tumor	**≤ 2 cm**	6 (37,5%)	3 (7,9%)	6 (20%)	0.005
	2 – 4 cm	9 (56,2%)	12 (31,6%)	13 (43,3%)	
	≥ 4 cm	1 (6,2%)*	23 (60,5%)	11 (36,7%)	
Infiltration depth vulva tumor	**1-4 mm**	5 (31,2%)	7 (18,4%)	10 (33,3%)	0.347
	≥ 4 mm	11 (68,8%)	31 (81,6%)	20 (66,7%)	
Focality vulva tumor	**Unifocal**	16 (100%)	27 (71,1%)	22 (73,3%)	0.369
	Multifocal	0 (0%)	11 (28,9%)	8 (26,7%)	
Pre-operative suspicious lymph nodes **	**No**	16 (100%)	4 (10,5%)	13 (43,3%)	< 0.001
	Yes	0 (0%)	34 (89,5%)	17 (56,7%)	
Number of removed lymph nodes (median and range)		1 (1-4)	3 (1-15)	8 (3-19)	< 0.001
Number of positive nodes		1,1	2,1 (1-12)	2,6 (1-19)	0.007
N stage ***	**N1**	12 (75%)	5 (13,2%)	7 (23,3%)	< 0.001
	N2	3 (18,8%)	32 (84,2%)	21 (70%)	
	N3	0 (0%)	0 (0%)	0 (0%)	
	Unknown	1 (6,2%)	1 (2,6%)	2 (6,7%)	
Size of groin metastases	**ITC**	5 (31,2%)	1 (2,6%)	0 (0%)	< 0.001
	≤ 2 mm	7 (43,8%)	0 (0%)	2 (6,7%)	
	>2 mm	4 (25%)****	36 (94,8%)	28 (93,3%)	
	Unknown	0 (0%)	1 (2,6%)	0 (0%)	
Nodal growth	**Intact**	13 (81,2%)	11 (29%)	16 (53,3%)	0.005
	Extra capsular	3 (18,8%)	26 (68,4%)	14 (46,7%)	
	Unknown	0 (0%)	1 (2,6%)	0 (0%)	
Adjuvant treatment groin metastasis	**None**	5 (31,2%)*****	1 (2,6%)	7 (23,3%)	0.005
	Radiotherapy	11 (68,8%)	34 (89,5%)	20 (66,7%)	
	Chemoradiation	0 (0%)	3 (7,9%)	3 (10%)	

Complication groin surgery		2 (12,5%)	5 (13,2%)	16 (53,3%)	0.003
Type of complication groin surgery	**Lymphocyst**	1 (6,2%)	0 (0%)	8 (26,7)	0.032
	Lymphedema	1 (6,2%)	0 (0%)	13 (43,3%)	0.002
	Wound dehiscence	0 (0%)	2 (5, 3%)	3 (10%)	0.649
	Wound infection	1 (6,2%)	1 (2,6%)	7 (23,3%)	0.091
	Hematoma	0 (0%)	2 (5,3%)	0 (0%)	0.765
Number of complications	**None**	14 (87,6%)	33 (86,9%)	14 (46,7%)	0.010
	One	1 (6,2%)	4 (10,5%)	9 (30%)	
	Two	1 (6,2%)	1 (2,6%)	2 (6,7%)	
	Three	0 (0%)	0 (0%)	5 (16,6%)	
Number of groin recurrences		4 (25%)	6 (15,8%)	4 (13,3%)	0.495
Mean time until groin recurrence (months)		9,3 (7-14)	17,3 (3,8-37)	5,8 (2,1-12,6)	0.601

* This patient underwent a SN procedure because the size of the vulva tumor turned out to be larger than clinically assessed. In the SN ITC's were found. After multidisciplinary consultation it was decided to treat this patient with postoperative radiotherapy instead of an IFL.
** Based on physical examination or pre-operative imaging
*** N stage according to the TNM classification of the American Cancer Society 2010. N1: the cancer has spread to 1 or 2 lymph nodes in the groin and the areas of cancer spread are both less than 5 mm in size or the cancer has spread to one lymph node and the area of cancer spread is 5 mm or greater. N2: the cancer has spread to 3 or more lymph nodes, but each area of spread is less than 5 mm or the cancer has spread to 2 or more lymph nodes with each area of spread 5 mm or greater or the cancer has spread to lymph nodes and has extracapsular spread in at least one lymph node. N3: the cancer has spread to the lymph nodes causing ulceration or causing the lymph node to be fixed to the tissue below it.
**** These four patients with a macrometastasis in the SN all underwent their SN procedure before 2010. Three patients received adjuvant radiotherapy and one patient refused radiotherapy. Before 2010 the SN procedure was not followed by IFL.
***** Five patients in the SN procedure group did not receive adjuvant radiotherapy. One patient with pre-existent cardiac disease died two weeks after surgery due to an acute myocardial infarction. Two patients refused adjuvant radiotherapy (one patient with a micrometastasis and one patient with a macrometastasis, both patients developed a groin recurrence). For two patients with one lymph node metastasis with ITC's it was decided in a multidisciplinary meeting that further treatment was not desirable because of their high age, comorbidity and the small chance on additional metastases in the non-sentinel nodes.

ITC: isolated tumor cells (considered as tumor positive lymph nodes)
SN: sentinel node
IFL: inguinofemoral lymphadenectomy

In 14 of the 84 groins a groin recurrence occurred. One patient with lymph node metastases in both groins treated with bilateral IFL and chemoradiation developed a groin recurrence in both groins. Four groin recurrences (25%) occurred in the sixteen groins which were initially treated with SN only. In two of these four cases no adjuvant radiotherapy was given because of refusal, one groin contained isolated tumor cells and was treated with adjuvant radiotherapy, and one groin contained a macrometastasis and was treated with adjuvant radiotherapy. Six groin recurrences (16%) occurred in the 38 groins treated with debulking and four groin recurrences (13%) in the 30 groins in which IFL was performed. The number of groin recurrences was not significantly different between the initial surgical modality groups (p=0.495). There was also no significant difference in groin recurrence-free time between the different surgical modality groups (p=0.904) (Figure 1).The mean time until recurrence was 9,3 months in the SN procedure group, 17,3 months in the debulking group, and 5,8 months in the IFL group (p=0.156). When the SN group was excluded, the difference between the number of groin recurrences and the mean time until groin recurrence remained non-significant.

Morbidity after groin surgery

The risk of complications after groin surgery was significantly lower in the debulking and SN groups compared to IFL (13% and 13% versus 53% , p=0.003). Lymphocysts and lymphedema occurred less often in the debulking and SN groups compared to the IFL group (0% and 6% versus 27% , p=0.032 and 0% and 6% versus 43%, respectively, p=0.002). In addition, significant more patients suffered from more than one complication in the IFL group than in the other treatment groups (p=0.010). The occurrence of lymphocysts, lymphedema and overall complication rate remained significantly higher in the IFL group compared to the debulking group (p=0.011, p=0.002, p=0.005, respectively) after excluding the SN group from the analysis.

Univariate and multivariate analysis

Univariate and multivariate analyses on groin recurrence-free time were performed in the 84 groins with lymph node metastases. In the univariate analysis we found no significant prognostic variables for groin recurrence-free time. Importantly, the type of primary surgical treatment of the groin was not significantly related to groin recurrence-free time. In multivariate analysis we also found no significant prognostic variables for groin recurrence-free time (Table 3). Again, initial surgical groin treatment modality, either SN, debulking or IFL, was not significantly related to groin recurrence-free time (p=0.650).

Table 3: Univariate and multivariate analysis of groin recurrence free time in 84 groins

	Univariate analysis		Multivariate analysis	
Variable	**Hazard (CI)**	**P-value**	**Hazard (CI)**	**P-value**
Primary groin treatment		0.905		0.650
IFL versus debulking	0.898 (0.253 – 3.193)	0.868	1.782 (0.447 – 7.102)	0.413
SN-procedure versus debulking	0.754 (0.344 – 4.371)	0.754	1.618 (0.449 – 5.826)	0.462
SN-procedure versus IFL	2.075 (0.529 – 10.989)	0.255	1.101 (0.259 – 4.683)	0.896
Age	0.984 (0.953 – 1.017)	0.339	0.976 (0.946 – 1.007)	0.127
FIGO stage	0.982 (0.273 – 3.530)	0.982		
Tumor size		0.903		
< 2 cm versus 2-4 cm	0.760 (0.203 – 2.849)	0.684		
2-4 cm versus >4 cm	1.257 (0.363 – 4.347)	0.717		
< 2 cm versus >4 cm	0.956 (0.253 – 3.607)	0.947		
LVSI (yes versus no)	0.174 (0.023 – 1.337)	0.093	0.131 (0.016 – 1.038)	0.054
Nodal growth (extracapsular versus intact)	1.734 (0.593 – 5.076)	0.315		
Depth of infiltration (>4 mm versus 1-4 mm)	3.681 (0.810 – 16.730)	0.092	4.447 (0.893 – 22.154)	0.069
Number of removed lymph nodes	1.063 (0.956 – 1.182)	0.256		
Number of tumor positive lymph nodes	1.052 (0.877 – 1.263)	0.585		
Size lymph node metastases		0.678		
ITC versus micrometastases	0.597 (0.037 – 9.592)	0.716		
ITC versus macrometastases	1.433 (0.186 – 11.045)	0.730		
Micrometastases versus macrometastases	2.398 (0.308 – 18.519)	0.403		
Adjuvant groin treatment		0.359		
No treatment versus radiotherapy	0.612 (0.164 – 2.280)	0.464		
No treatment versus chemoradiation	1.778 (0.296 – 10.688)	0.529		
Radiotherapy versus chemoradiation	2.906 (0.625 – 13.513)	0.174		

IFL: inguinofemoral lymphadenectomy
SN: sentinel node
LVSI: lymphovascular space invasion

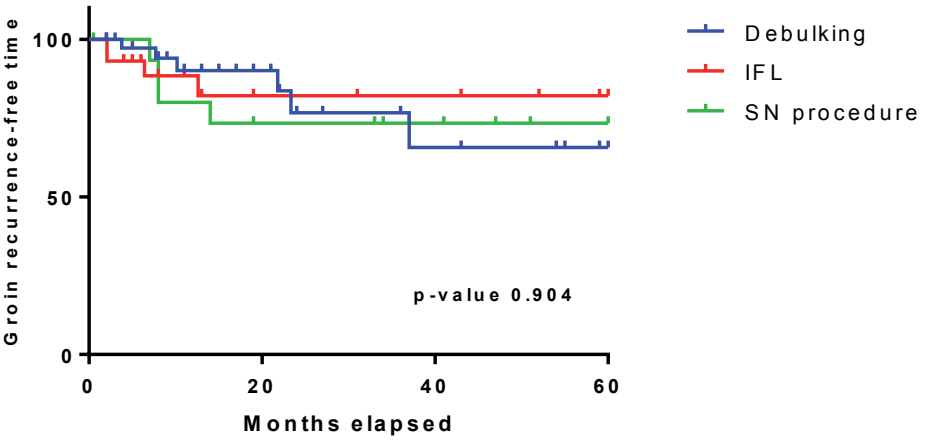


Figure 1: groin recurrence free time in 84 groins of 68 patients who had primary surgical treatment of lymph node positive groins.

IFL: inguinofemoral lymphadenectomy
SN: sentinel node

Discussion

Most patients with VSCC and proven metastases to the groin nodes are treated with extensive IFL surgery and adjuvant radiotherapy or chemoradiation, resulting in high morbidity rates. Thus far, there is no consensus whether groin metastases can best be treated with debulking surgery of clinically involved or enlarged nodes followed by radiotherapy, or with more radical removal of all inguinofemoral lymph nodes (IFL), with radiotherapy when indicated (30). In this study we analysed whether the type of initial surgical procedure of the groin influenced groin recurrence-free time in patients with VSCC and cytologically or histologically proven groin metastases. Furthermore, we analysed the negative impact of a groin recurrence on overall survival, and compared the morbidity of the different initial surgical groin treatment modalities. Our results show that patients with a groin recurrence have a nine times higher risk of dying of disease compared to patients who do not develop a groin recurrence. These findings emphasise the importance of obtaining groin control at first treatment. Because VSCC patients are often fragile and elderly, it is also of major importance to choose a treatment modality with the lowest risk of morbidity and complications. We found that there was no significant difference regarding the risk of groin recurrence between the initial surgical treatment groups with proven lymph node metastases. Furthermore, it was shown that both debulking surgery and SN procedure had a significantly lower complication rate compared to IFL. Especially the risk of developing lymphocysts or lymphedema was

significantly lower after debulking or SN procedure compared to IFL, regardless of postoperative radiotherapy.

The SN procedure has led to a major decrease in morbidity compared to IFL without influencing prognosis (8, 19, 37-39). However, this procedure is only suitable and reliable in early stage vulvar cancer patients with unifocal lesions, tumor size less than 4 cm and clinically negative lymph nodes (1, 8, 21). Therefore, a selected group of patients still needs extensive surgery of the groins, consisting of either IFL or debulking of clinically involved lymph nodes followed by radiotherapy. The preferred treatment of proven metastases to the groin, especially in case of macrometastases >2 mm, remains to be answered. In a retrospective study, Hyde et al. (30) compared IFL with nodal debulking regarding groin recurrence and survival in forty patients with VSCC and clinically involved groin nodes. They found no difference in groin recurrence rate, and concluded that nodal debulking does not jeopardize survival in comparison to IFL when both are followed by groin and pelvic radiation. These results are confirmed by our study. We did not find a significant difference in groin recurrence rate when only comparing the debulking group with the IFL group. This is especially important because these groups are more homogenous in contrast to the SN group (Table 2), and reflect patients with more advanced disease. In addition to the study of Hyde et al. we also analysed postoperative morbidity and found that postoperative morbidity was worse in patients who received IFL, as also found in a previous study (16). We found a complication rate as low as 13% in patients who underwent debulking compared to 53% in patients who underwent IFL (Table 2). Even despite the fact that significant more patients received adjuvant radiotherapy in the debulking group, which is regarded to be associated with a higher complication rate.

Although we confirmed the results of Hyde et al. in a larger series of patients and supplemented our results with morbidity rates, our study group remains relatively small. This remains a limitation for all studies on VSCC because of the rarity of the disease. Also, retrospective analyses have the inherent limitations of differences in treatment selection and outcomes over time. Diagnostic and therapeutic procedures have significantly improved, and have likely increased the rate of patients treated to all involved and clinically relevant lymph node regions with radiotherapy. Radiotherapy techniques have improved both in terms of dose distribution, accuracy, and reduction of late complications by use of more conformal, and in later years also image-guided, intensity modulated treatment techniques. This could have led to better outcomes in more recent years, and contributed to the lower rate of complications in the debulking group as compared to IFL despite the more frequent use of radiotherapy (98 vs 77%).

The treatment of patients with VSCC and proven groin metastases remains a clinical challenge, as these patients need additional treatment in order to improve their prognosis. With the current treatment modalities, prognosis of patients with proven lymph node metastases is still poor while treatment-associated morbidity rates are high. For patients with clinically suspicious inguinofemoral lymph nodes and/or macrometastases >2 mm our findings suggest that nodal debulking followed by radiotherapy is the preferred mode of treatment. Debulking of pathologic or enlarged nodes is related to a lower risk of complications, also in combination with postoperative radiotherapy, without increasing the risk of recurrence, compared to IFL. These findings are a relevant contribution to the growing body of data that will help to individualize the surgical treatment of patients with VSCC. Because of the low incidence of node-positive vulvar cancer, larger, prospective studies are needed.

Conflict of interest statement

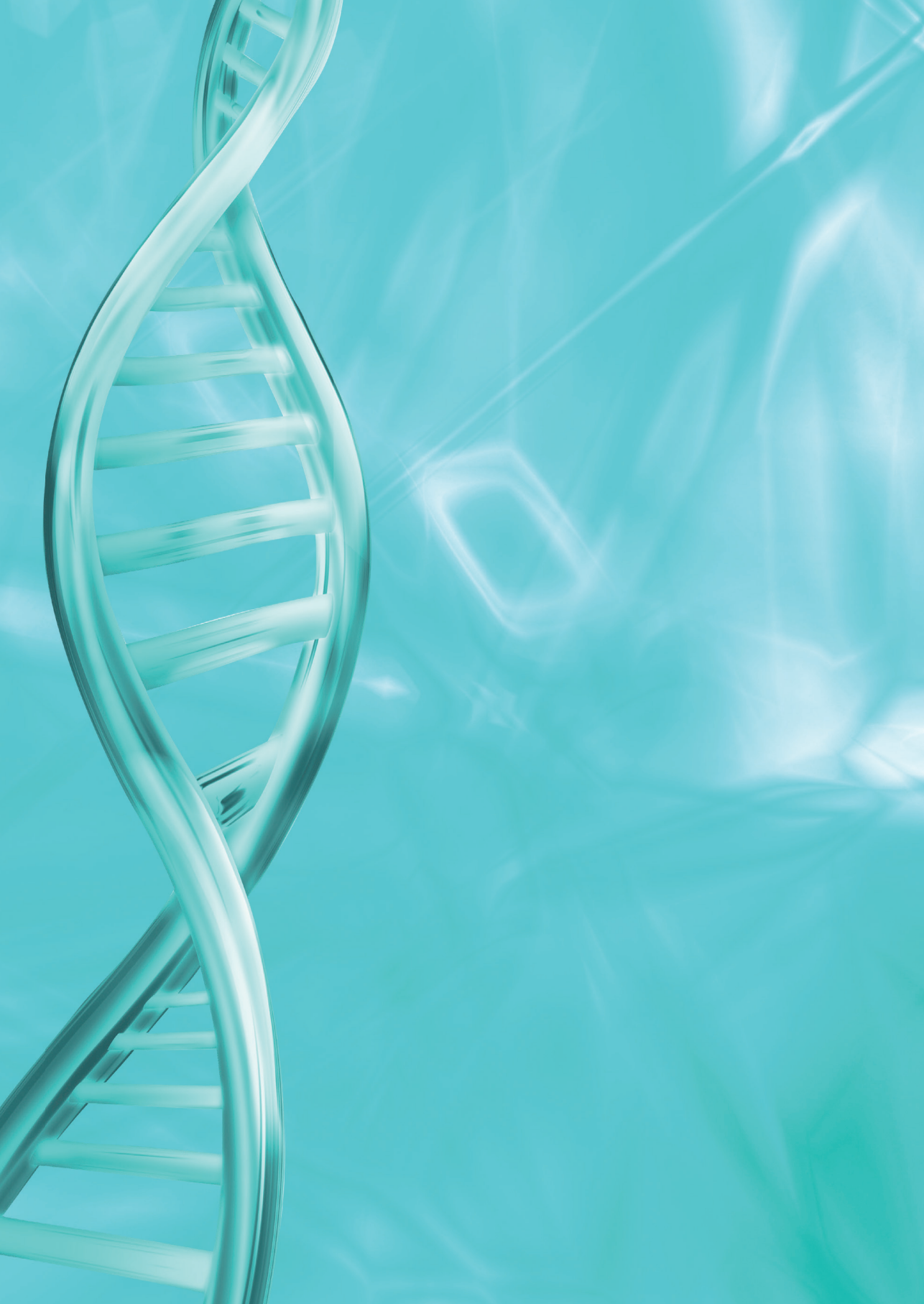
There are no conflicts of interest.

References

1. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.
2. Burger MP, Hollema H, Emanuels AG, Krans M, Pras E, Bouma J. The importance of the groin node status for the survival of T1 and T2 vulval carcinoma patients. GynecolOncol. 1995;57(3):327-34.
3. Gadducci A, Cionini L, Romanini A, Fanucchi A, Genazzani AR. Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer. Crit RevOncolHematol. 2006;60(3):227-41.
4. Aragona AM, Cuneo NA, Soderini AH, Alcoba EB. An analysis of reported independent prognostic factors for survival in squamous cell carcinoma of the vulva: is tumor size significance being underrated? GynecolOncol. 2014;132(3):643-8.
5. de Hullu JA, van der Zee AG. Surgery and radiotherapy in vulvar cancer. Crit RevOncolHematol. 2006;60(1):38-58.
6. Konidaris S, Bakas P, Gregoriou O, Kalampokas T, Kondi-Pafiti A. Surgical management of invasive carcinoma of the vulva. A retrospective analysis and review. EurJGynaecolOncol. 2011;32(5):505-8.
7. Tabbaa ZM, Gonzalez J, Sznurkowski JJ, Weaver AL, Mariani A, Cliby WA. Impact of the new FIGO 2009 staging classification for vulvar cancer on prognosis and stage distribution. GynecolOncol. 2012;127(1):147-52.
8. van der Zee AG, Oonk MH, de Hullu JA, Ansink AC, Vergote I, Verheijen RH, et al. Sentinel node dissection is safe in the treatment of early-stage vulvar cancer. JClinOncol. 2008;26(6):884-9.
9. de Hullu JA, van der Zee AG. Groin surgery and the sentinel lymph node. BestPractResClinObstetGynaecol. 2003;17(4):571-89.
10. van Beekhuizen HJ, Auzin M, van den Einden LC, de Hullu JA, van d, V, Wildhagen MF, et al. Lymph node count at inguinofemoral lymphadenectomy and groin recurrences in vulvar cancer. IntJGynecolCancer. 2014;24(4):773-8.
11. Deka P, Barmon D, Shribastava S, Kataki AC, Sharma JD, Bhattacharyya M. Prognosis of vulval cancer with lymph node status and size of primary lesion: A survival study. JMidlifeHealth. 2014;5(1):10-3.
12. Oonk MH, Hollema H, de Hullu JA, van der Zee AG. Prediction of lymph node metastases in vulvar cancer: a review. IntJGynecolCancer. 2006;16(3):963-71.
13. Cormio G, Loizzi V, Carriero C, Cazzolla A, Putignano G, Selvaggi L. Groin recurrence in carcinoma of the vulva: management and outcome. EurJCancer Care (Engl). 2010;19(3):302-7.
14. Hopkins MP, Reid GC, Morley GW. The surgical management of recurrent squamous cell carcinoma of the vulva. Obstet Gynecol. 1990;75(6):1001-5.
15. Salom EM, Penalver M. Recurrent vulvar cancer. Curr TreatOptions Oncol. 2002;3(2):143-53.
16. Gaarenstroom KN, Kenter GG, Trimbos JB, Agous I, Amant F, Peters AA, et al. Postoperative complications after vulvectomy and inguinofemoral lymphadenectomy using separate groin incisions. IntJGynecolCancer. 2003;13(4):522-7.
17. Hacker NF. Current management of early vulvar cancer. AnnAcadMedSingapore. 1998;27(5):688-92.
18. Saito T, Kato K. Management of lymph nodes in the treatment of vulvar cancer. IntJClinOncol. 2007;12(3):187-91.

19. Oonk MH, van Hemel BM, Hollema H, de Hullu JA, Ansink AC, Vergote I, et al. Size of sentinel-node metastasis and chances of non-sentinel-node involvement and survival in early stage vulvar cancer: results from GROINSS-V, a multicentre observational study. Lancet Oncol. 2010;11(7):646-52.
20. Velden vd, Fons G, Lawrie TA. Primary groin irradiation versus primary groin surgery for early vulvar cancer. CochraneDatabaseSystRev. 2011(5):CD002224.
21. Levenback CF, Ali S, Coleman RL, Gold MA, Fowler JM, Judson PL, et al. Lymphatic mapping and sentinel lymph node biopsy in women with squamous cell carcinoma of the vulva: a gynecologic oncology group study. JClinOncol. 2012;30(31):3786-91.
22. de Hullu JA, Oonk MH, van der Zee AG. Modern management of vulvar cancer. CurrOpinObstetGynecol. 2004;16(1):65-72.
23. Woelber L, Eulenburg C, Choschzick M, Kruell A, Petersen C, Gieseking F, et al. Prognostic role of lymph node metastases in vulvar cancer and implications for adjuvant treatment. IntJGynecolCancer. 2012;22(3):503-8.
24. Schuurman MS, van den Einden LC, Massuger LF, Kiemeney LA, van der Aa MA, de Hullu JA. Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma. EurJCancer. 2013;49(18):3872-80.
25. van de Nieuwenhof HP, Massuger LF, van der Avoort IA, Bekkers RL, Casparie M, Abma W, et al. Vulvar squamous cell carcinoma development after diagnosis of VIN increases with age. EurJCancer. 2009;45(5):851-6.
26. Hinten F, van den Einden LC, Hendriks JC, van der Zee AG, Bulten J, Massuger LF, et al. Risk factors for short- and long-term complications after groin surgery in vulvar cancer. BrJCancer. 2011;105(9):1279-87.
27. Berger J, Scott E, Sukumvanich P, Smith A, Olawaiye A, Comerci J, et al. The effect of groin treatment modality and sequence on clinically significant chronic lymphedema in patients with vulvar carcinoma. IntJGynecolCancer. 2015;25(1):119-24.
28. Petereit DG, Mehta MP, Buchler DA, Kinsella TJ. Inguinofemoral radiation of N0,N1 vulvar cancer may be equivalent to lymphadenectomy if proper radiation technique is used. IntJRadiatOncolBiolPhys. 1993;27(4):963-7.
29. Wills A, Obermair A. A review of complications associated with the surgical treatment of vulvar cancer. GynecolOncol. 2013;131(2):467-79.
30. Hyde SE, Valmadre S, Hacker NF, Schilthuis MS, Grant PT, van d, V. Squamous cell carcinoma of the vulva with bulky positive groin nodes-nodal debulking versus full groin dissection prior to radiation therapy. IntJGynecolCancer. 2007;17(1):154-8.
31. Desimone CP, Elder J, van Nagell JRJ. Selective inguinal lymphadenectomy in the treatment of invasive squamous cell carcinoma of the vulva. IntJSurgOncol. 2011;2011:284374.
32. Woelber L, Kock L, Gieseking F, Petersen C, Trillsch F, Choschzick M, et al. Clinical management of primary vulvar cancer. EurJCancer. 2011;47(15):2315-21.
33. Hutteman M, van der Vorst JR, Gaarenstroom KN, Peters AA, Mieog JS, Schaafsma BE, et al. Optimization of near-infrared fluorescent sentinel lymph node mapping for vulvar cancer. AmJObstetGynecol. 2012;206(1):89-5.

34. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
35. Baiocchi G, Silva Cestari FM, Rocha RM, Lavorato-Rocha A, Maia BM, Cestari LA, et al. Prognostic value of the number and laterality of metastatic inguinal lymph nodes in vulvar cancer: revisiting the FIGO staging system. EurJSurgOncol. 2013;39(7):780-5.
36. Woelber L, Mahner S, Voelker K, Eulenburg CZ, Gieseking F, Choschzick M, et al. Clinicopathological prognostic factors and patterns of recurrence in vulvar cancer. Anticancer Res. 2009;29(2):545-52.
37. Moore RG, Robison K, Brown AK, DiSilvestro P, Steinhoff M, Noto R, et al. Isolated sentinel lymph node dissection with conservative management in patients with squamous cell carcinoma of the vulva: a prospective trial. GynecolOncol. 2008;109(1):65-70.
38. de Hullu JA, Hollema H, Piers DA, Verheijen RH, van Diest PJ, Mourits MJ, et al. Sentinel lymph node procedure is highly accurate in squamous cell carcinoma of the vulva. JClinOncol. 2000;18(15):2811-6.
39. Johann S, Klaeser B, Krause T, Mueller MD. Comparison of outcome and recurrence-free survival after sentinel lymph node biopsy and lymphadenectomy in vulvar cancer. GynecolOncol. 2008;110(3):324-8.



CHAPTER 4

Risk factors and treatment for recurrent vulvar squamous cell carcinoma

Linda S. Nooij
Fernande A.M. Brand
Katja N. Gaarenstroom
Carien L. Creutzberg
Joanne A. de Hullu
Mariette I.E. van Poelgeest

Critical Reviews in Oncology/Hematology 2016;106:1-13

Abstract

Recurrent disease occurs in 12–37% of patients with vulvar squamous cell carcinoma (VSCC). Decisions about treatment of recurrent VSCC mainly depend on the location of the recurrence and previous treatment, resulting in individualized and consensus-based approaches. Most recurrences (40–80%) occur within 2 years after initial treatment. Currently, wide local excision is the treatment of choice for local recurrences. Isolated local recurrence of VSCC has a good prognosis, with reported 5-year survival rates of up to 60%. Groin recurrences and distant recurrences are less common and have an extremely poor prognosis. For groin recurrences, surgery with or without (chemo)radiotherapy is a treatment option, depending on prior treatment. For distant recurrences, there are only palliative treatment options. In this review, we give an overview of the available literature and discuss epidemiology, risk factors, and prognostic factors for the different types of recurrent VSCC and we describe treatment options and clinical outcome.

1. Introduction

Vulvar cancers account for 3–5% of all gynecological malignancies, with an annual incidence of 1–2 per 100,000 women (1-4). The incidence of vulvar cancer increases with age, with a peak incidence in the seventh decade (1, 3). The overall incidence of vulvar cancer has risen over the last decade, probably because of an increase in human papilloma virus (HPV) infections and higher life expectancy (5). Around 80–90% of these tumors are squamous cell carcinomas. Malignant melanoma, Bartholin gland carcinoma, invasive Paget's disease, and basal cell carcinoma are less frequent. Other tumor types, such as sarcomas and verrucous carcinomas, are extremely rare (1-3).

Five-year survival for early-stage VSCC is about 80–90% (1, 6). Prognosis is strongly dependent on the presence of lymph node metastases (3, 6-9). Therefore, the International Federation of Gynecology and Obstetrics (FIGO) staging system was changed in 2009 (Table 1) (10). Tumors with negative lymph node status can be regarded as low risk, regardless of tumor diameter and expansion to the vagina and/or urethra. By contrast, the number, size, and extranodal growth of involved lymph nodes are important prognostic factors. An increasing number of positive lymph nodes, a larger diameter of nodal metastases, and extranodal growth are significantly related to worse survival (11).

Table 1: FIGO staging system of vulvar cancer

Stage	
I	Tumours confined to the vulva or perineum, no nodal metastasis Ia: Tumour ≤ 2 cm with stromal invasion ≤ 1 mm Ib: Tumour > 2 cm or stromal invasion > 1mm
II	Tumour of any size with extension to adjacent perineal structures (lower urethra, lower vagina, anus), no nodal metastasis
III	Tumour of any size with or without extension to adjacent perineal structures (lower urethra, lower vagina, anus), with inguino-femoral nodal metastasis IIIa: 1 node metastasis (≥ 5 mm) or 1-2 node metastasis(es) (< 5 mm) IIIb: ≥ 2 node metastases (≥ 5 mm) or ≥ 3 node metastases (< 5 mm) IIIc: node metastases with extra-capsular spread
IV	Iva: Tumour invades any of the following: upper urethra and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to pelvic bone, or fixed or ulcerated inguinofemoral nodes IVb: Any distant metastasis including pelvic nodes

Carcinogenesis of VSCC can be subdivided into two different pathways. One pathway is associated with lichen sclerosus (LS) and usually occurs in older patients (55–85 years) (4, 12-18). This pathway accounts for around 70% of all VSCC. Differentiated vulvar intraepithelial neoplasia (dVIN) is the presumed precursor lesion found in this type of VSCC. It has been suggested that untreated dVIN has a high malignant potential, probably as high as 80% (19). The other known pathway is human papilloma virus (HPV) dependent and accounts for around 30% of all VSCC. The most prevalent HPV

types found in VSCC are HPV16 in 60–78% of cases followed by HPV18 in 5–16% (13, 14, 16-18, 20-25). This pathway usually occurs in younger patients (35–65 years) and is associated with vulvar high grade squamous intraepithelial lesions (HSIL, formerly referred to as usual type VIN) and smoking. Untreated vulvar HSIL has a lower rate of progression to VSCC (9–16%) (15, 19) compared to dVIN. Although most VSCC are HPV independent, dVIN accounts for only 2–10% of all reported VIN lesions (15, 25). The low prevalence of dVIN may be explained by the belief that it progresses rapidly to VSCC. Another explanation may be that dVIN is an underdiagnosed and therefore underreported lesion due to its subtle clinical and histological features. Although dVIN has been described already in 1961 by Abell et al. (26), it is only recently that dVIN has been recognized and regarded as a distinctive diagnosis by clinicians as well as pathologists (15). Recently, the International Society for the Study of Vulvar Disease (ISSVD) published a new classification system for VIN. The new terminology discriminates between HPV-dependent low-grade squamous intraepithelial lesions (LSIL; i.e., flat condyloma or HPV effect) and high-grade squamous intraepithelial lesions (HSIL) on the one hand, and the HPV-independent precursor dVIN on the other (Table 2) (27). Because precursor lesions are frequently found in the presence of VSCC, clinicians should take the phenomenon of "field cancerization" into account: the majority of "recurrences" maybe considered "de novo" tumors in a background of epithelial changes already at risk for the development of malignancy (19, 28, 29).

Table 2: Old and new terminology of vulvar squamous intraepithelial lesions

ISSVD 1986	ISSVD 2004	ISSVD 2015
VIN 1	Flat condyloma or HPV effect	LSIL
VIN 2 VIN 3	VIN, usual type (uVIN)	HSIL
Differentiated VIN (dVIN)	VIN, differentiated type (dVIN)	VIN, differentiated type (dVIN)

ISSVD: International Society for the Study of Vulvovaginal Disease
VIN: Vulvar Intraepithelial Neoplasia
LSIL: Low grade squamous intraepithelial lesion
HSIL: High grade squamous intraepithelial lesion

Surgery is the cornerstone of treatment for primary VSCC (1, 2, 4). Surgery for tumors infiltrating >1 mm generally consists of wide local excision with full uni- or bilateral inguinofemoral lymphadenectomy (IFL) or sentinel lymph node (SLN) biopsy. A full IFL is defined as the surgical removal of all lymph node–bearing fatty tissue of the superficial inguinal and deep femoral loge medial to the fossa ovalis. SLN biopsy is considered safe in a selected group of patients with VSCC: those with a unifocal vulvar tumor <4 cm without enlarged or clinically suspected groin lymph nodes upon palpation

and imaging (30). Adjuvant radiation therapy is indicated for close or involved surgical margins and lymph node involvement depending on the size and number of nodal metastases and the presence of extranodal growth. Concurrent chemoradiotherapy in a neoadjuvant setting is recommended, especially for downsizing of bulky disease, in particular when the urethra or anus are involved (2, 3, 30-33). Despite these treatment modalities, recurrence rates are still high: 12–37% (7, 34). Furthermore, prognosis of patients with recurrent VSCC has not improved over the past decades, with a reported 5-year survival rate of 25–50% (34-38).

There are several challenges in the treatment of recurrent VSCC. Most VSCC patients are over 60 years of age, with significant comorbidity. Moreover, treatment of recurrent VSCC is associated with a high risk of developing complications (5, 25). The choice of treatment for recurrent VSCC is determined by the localization of recurrence and prior treatment (8, 34, 39). Nevertheless, the literature is relatively scarce and clear guidelines for the treatment of recurrent VSCC are lacking (40-42). In this review, we present an overview of the available literature on known risk factors for recurrence and treatment options for recurrent VSCC, including associated morbidity and clinical outcome.

1.1 Data sources

We performed an extensive search on PubMed, Embase, Web of Science, Cochrane, and ScienceDirect. After consulting a medical librarian, we formulated a combination of Medical Subject Headings (MeSH) and free text words. Our search included the terms vulvar neoplasm, vulvar carcinoma, vulvar neoplasia, groin, metastasis, and recurrence. The electronic search was complemented by a manual search of reference lists for relevant publications. In addition, we collected information from national and international oncological guidelines and checked study books for further data (4, 40-44).

A total of 1303 articles were identified. All articles were assessed by two independent authors (LN and FB) on title, abstract, or full article. Inclusion criteria were original articles that reported on treatment of recurrent VSCC (either local, groin or distant recurrences). To avoid inclusion of too small studies we chose to only report studies that included a minimum of respectively 20 or 10 patients (local or groin recurrence). After exclusion of articles based upon title and abstract 67 articles remained of which the full article was judged. Finally, twenty-four articles met our inclusion criteria and were included in this review.

1.2 Terminology

There is no clear definition of local VSCC recurrence in the current literature. There is no consensus on the minimum or maximum time span until local recurrence and on the distance between recurrent disease and the initial or primary vulvar tumor. Some authors define local recurrence as the new appearance of a tumor after therapy with radical intent

and a disease-free period of at least 6 months (45). Rouzier et al. defined three patterns of local recurrence: primary tumor site recurrence, remote vulvar recurrence (>2 cm from the primary tumor site), and skin bridge recurrence (9). Because documentation of the exact location of the primary tumor is often equivocal, the distance to the primary site to define recurrence is difficult to assess. In ongoing and future prospective studies, introduction of digital cameras may be helpful in reporting the exact location of the tumor. Another point of discussion addresses the time span that should be used to define tumor recurrence versus de novo tumor. Most recurrences occur within 2 years after primary treatment, so recurrences after 2–3 years might be considered de novo tumors (34, 46).

For this review, we defined a local recurrence as a "new or de novo" tumor on the vulva after primary treatment of VSCC, irrespective of location on the vulva, distance from the primary tumor, or time interval from initial therapy to recurrence. We stand by this definition because differences in these features were not described clearly in the available literature. A groin recurrence is defined as any (recurrent) lymph node metastasis in the groin(s) with or without the presence of a local recurrence after initial treatment for VSCC. A skin bridge recurrence is considered a special type of locoregional recurrence and is defined as a new tumor in the dermis in the intervening skin between the operated-on vulva and the ipsilateral groin region. Distant or metastatic recurrence is defined as any recurrence beyond the vulva or groins, whether or not asynchronous with a local or groin recurrence. Pelvic recurrences are considered distant recurrences.

2. Epidemiology and risk factors

Recurrences of VSCC occur in 12–37% of patients after initial treatment, depending on tumor stage at initial diagnosis (7, 34); 40–80% of all recurrences occur within 2 years of initial treatment (1, 34). Outcome for patients who had recurrences within 2 years after initial surgery is worse compared to patients who had recurrences >2 years after initial treatment (8, 47). In a prospective study of 143 patients with VSCC, Stehman et al. found a median time until local recurrence of 35.9 months: 19% of the VSCC recurred in the first year after therapy, and 28.6% recurred in the first 2 years after therapy. All patients included in this study underwent surgery to remove the local tumor, consisting of a modified radical hemivulvectomy or radical vulvectomy. Primary groin treatment consisted of superficial inguinal lymphadenectomy in 120 patients and groin irradiation in 23 patients (38). Another study identified local recurrences in the first year after treatment in 39% of patients, with an equal distribution during the following years (45). A recently published study reporting long term follow-up data of the GROINSS-VI study in patients with unifocal VSCC found a local recurrence rate of 27% after 5 years, with a median time to local recurrence of 33 months (range 2–128 months). The reported local recurrence rate at 10 years was as high as 40%. This 'recurrence' rate after 10 years was even higher than expected later in the course of disease (48). Mean follow-up after initial treatment was shorter in most previous studies compared to the long-term follow-up of the GROINSS-VI study. This might have underestimated the true incidence of recurrent disease. Furthermore, it still can be argued whether recurrent disease after several years must be regarded as true recurrence or de novo disease. Because of late "recurrences", several guidelines advise lifelong follow-up after treatment for VSCC (41, 42). Routinely scheduled follow-up leads to detection of smaller local recurrences in a considerable proportion of patients (49). The median time until recurrence in the groin is 7 months. The majority of the groin recurrences (67–73%) occur in the first year after initial treatment (38, 45, 48). Distant recurrences predominantly occur within 2 years after initial treatment (45, 48).

2.1 Local recurrence

The incidence of isolated local recurrences is 20–23% (45, 48, 50). More than 50% of all recurrences are local (7, 8, 34, 38, 45, 51); they are mainly isolated or associated with the groin or distant recurrences (7, 38, 45, 51). Univariate risk factors for local recurrence are higher age (50, 51), greater tumor size (34, 51-53), a multifocal tumor (52), depth of invasion >2 mm (9, 51, 54, 55), lymphovascular space invasion (52), and the presence of lymph node metastases at initial treatment (6, 45, 51). Except for greater tumor size, all of these risk factors are independent risk factors for local recurrence in multivariate analyses. A recent study identified the presence of perineural invasion as an independent pathological risk factor for local recurrence (56). The presence of lymph

node metastases at initial treatment may reflect a more aggressive biological behavior of the tumor and therefore also a poor prognostic factor for local recurrence. Few studies have reported on the presence of precursor lesions as risk factors for local recurrence. Two studies found that LS is a risk factor for recurrent disease (50, 57). One study compared the presence of associated HSIL as a prognostic factor for recurrence with the absence of associated HSIL (relative risk 2.30, $p<0.019$) (52). Another study found that the presence of HSIL in the surgical margins resulted in a 3-fold higher risk of recurrence ($p=0.03$) (58). The width of the tumor-free margin status is one of the most clinical important and controversial topics in vulvar cancer treatment. Although it is obvious that a tumour positive margin is associated with an increased local recurrence rate, the association of the width of the tumor-free margin and local recurrence rate is less clear (46, 59-63). Heaps et al. (59) found that increasing tumor-free margins were associated with a decrease in the local recurrence rate in a group of 135 patients. In patients with a tumor-free margin of <8 mm, there was a 48% risk of local recurrence, compared to a 0% local recurrence rate for patients with a tumor-free margin of >8 mm (59). However, subsequent studies yielded varying results with regard to the tumor-free margin and the risk of local recurrence (46, 60-63). Moreover, in most of these studies, the difference between "true recurrences" and "de novo" tumors was not taken into account. Future studies should focus on investigating the optimal tumor-free margin for prevention of local recurrences.

2.2 Groin recurrence

In 9–38% (average 22%) of patients with recurrent VSCC, the groin is the site of recurrence (6, 34, 38, 50, 51, 64-66). Patients with lymph node involvement at initial diagnosis have a higher risk of developing a groin recurrence (6, 38, 50, 51, 64, 66). In patients with negative lymph nodes at initial diagnosis, the groin recurrence rate is estimated to be extremely low (0–2%), while for patients with positive lymph nodes, this risk is as high as 29–40% (30, 51, 64, 65). SLN procedure of the groin is the preferred staging in patients with unifocal disease <4 cm without enlarged or clinically suspected lymph nodes (30). In all other primary cases and local recurrences without earlier IFL a full IFL should be performed. The risk of groin recurrence can be reduced considerably with adjuvant radiotherapy to the inguinofemoral and pelvic region (3, 67-69). Because radiotherapy was not always applied routinely in case of positive nodes, in particular in older studies, the groin recurrence risk could be overestimated compared to today's standard treatment. Adjuvant radiotherapy is recommended in patients with lymph node involvement when there are two or more positive lymph nodes or in case of extracapsular extension (3).

The number of positive lymph nodes is also a strong risk factor for groin recurrences (7, 62, 65, 70, 71). Hacker et al. found that patients with more than three positive lymph

nodes had a 33% risk of groin recurrence, compared to 2.9% for patients with less than three positive lymph nodes (70). Three studies described the impact of the number of removed lymph nodes at IFL on groin recurrence rate, with higher risk of groin recurrences and/or poorer survival after removal of less than 9–12 lymph nodes (72-74). However, it should be taken into account that removal of more lymph nodes and/or ultrastaging will lead to the identification of more and possibly otherwise-undetected lymph node metastases (72, 75). This could be a possible source of bias in the reported results. Other prognostic clinicopathological variables for groin recurrences are advanced FIGO stage (50), size of the lymph node metastases (7, 31, 76), and extracapsular nodal spread (7, 54, 71). In addition, groin treatment limited to superficial groin node dissection instead of a full IFL is associated with an increase in groin recurrences (77). The SLN procedure has been proven safe in these patients (403 patients in the GROINSS-V-I study), with a recurrence rate of 2.3% in patients with a negative SLN (30). In addition, the risk of groin recurrence is associated with the size of lymph node metastasis. Oonk et al. found lower disease-specific survival for patients with SLN metastases >2 mm (69.5%) compared to patients with SLN metastases ≤2 mm (94.4%, p=0.001) (31).

2.3 Distant recurrence

Distant recurrences are found in approximately 8% of patients with recurrent disease, and most distant recurrences occur within the first 2 years after treatment (84%) (6, 34, 45). The most common distant recurrence is pelvic recurrence (5–19% of the recurrences), which nearly always occurs together with a groin recurrence. Incidence of pelvic recurrence is dependent on treatment strategy as was shown by Homesley et al (68). In this study, 114 eligible patients with VSCC and positive groin nodes after radical vulvectomy and bilateral lymphadenectomy were randomized to receive either radiation therapy or pelvic node resection. In the radiotherapy group 68% remained free of recurrence, and rates of groin and pelvic recurrence were 5.1 and 6.8%, respectively. In the pelvic node dissection group, 55% remained recurrence-free, while rates of groin and pelvic recurrence were 23.6 and 1.8%. The estimated two-year survival rates were 68% for the radiation therapy group and 54% for pelvic node resection group (68). Multiple-site recurrences are described in about 14% of patients with recurrent disease (34, 45). Advanced stage of disease is a risk factor for the development of distant as well as multiple-site recurrences (7, 45, 50, 66).

2.4 HPV as a risk factor

In the last few years, more data have become available on the role of HPV in carcinogenesis. Infections with HPV have been linked to the development of vulvar, vaginal, cervical, anal and head and neck cancer, especially oropharyngeal malignancies (78-82). Like squamous cell carcinoma of the head and neck, VSCC can be subdivided into two different types: HPV independent and HPV dependent (13, 17, 19). For head

and neck carcinomas, it has become clear that these two types are clinically distinct with regard to response to treatment and survival outcome, with HPV positivity as a favorable prognostic biomarker (78-80).

There are conflicting data about the role of HPV status as a prognostic factor in VSCC. An overview of studies on the presence of HPV and impact on prognosis is given in table 3 (13, 16-18, 20, 22-25). The presence of HPV DNA can be detected by PCR (sequencing or INNO-LiPA) or in situ hybridization. HPV-independent VSCC are more common and seem to have a higher recurrence rate (mean 44%) and worse overall survival (mean 60%) compared to HPV-dependent VSCC (mean 26% and 78%, respectively), although no definitive conclusions can be drawn because of varying results and different definitions of HPV positivity (16, 17, 20, 22). VSCC associated with HSIL have a better prognosis with regard to local recurrence, disease-free survival, and overall survival compared to VSCC associated with LS and/or dVIN (15, 25, 83). One possible explanation for the prognostic difference between HPV-dependent and HPV-independent VSCC might be due to a better response to treatment of HPV-dependent cancers as has been shown in head and neck squamous cell carcinoma (78, 79). Of the studies included in table 3, only the study of Alonso et al. reported on the specific treatments in HPV+ and HPV- VSCC and found no differences between the two groups (13).

2.5 Prognosis

In general, 5-year survival for recurrent VSCC is reported to be 25–50% compared to 50–90% for patients with primary VSCC (40, 47, 52, 84, 85). Prognosis is mainly influenced by the presence of groin metastases at initial diagnosis. In addition, age, comorbidity, advanced FIGO stage, and tumor characteristics are important factors for prognosis and outcome (8, 9, 45, 46, 48, 50, 51, 64, 76, 84, 86). More recently, morphological factors such as spindle cell morphology and molecular changes, especially mutations in HRAS, were shown to be associated with poor prognosis in VSCC (87-89). Furthermore, prognosis is influenced by the site of recurrence and the time interval between initial diagnosis and recurrent disease. Five-year survival after diagnosis of recurrent disease for patients with early local recurrence (<24 months after primary treatment) was 53%, compared to 76% for patients with late local recurrence (>24 months after primary treatment) (p=0.05) (48, 90). The prognosis for patients with groin and/or skin bridge recurrence of VSCC is very poor with 5-year survival rates of only 0–10% (34-36, 50, 85, 91-94). However, a recently published study found an overall survival rate of 50% after 7 years for patients with a groin recurrence (95).

Table 3: Prevalence of HPV in VSCC and influence of HPV presence on prognosis

Author	N° of patients	N° HPV positive	N° HPV negative	Clinical outcome HPV positive	Clinical outcome HPV negative	Clinical outcome p-value
Larsson et al. 2012[22]	130	31%	69%	RR 30% OS 67%	RR 44% OS 43%	RR p = 0.121 OS **p = 0.029**
Alonso et al. 2011[13]	98	19%	81%	RR 60% OS 67%	RR 50% OS 71%	RR p = 0.885. Number of local recurrences lower in HPV positive VSCC (16% vs 37%, **p = 0.049**) OS p = 0.789
Lindell et al. 2010[16]	75	31%	69%	RR NS OS 100%	RR NS OS 65%	RR HPV positive better disease free survival; **p = 0.004** OS **p = 0.001**
Van de Nieuwenhof et al. 2009[19]	130	35%	65%	RR NS OS NS	RR NS OS NS	RR NS OS p = 0.646
Pinto et al. 2004[23]	161	24%	76%	RR 50% OS 63%	RR 35% OS 71%	RR p = 0.055 OS p = 0.447
Rouzier et al. 2001*[83]	77	32% vulvar HSIL adjacent to the VSCC	68% LS or dVIN adjacent to the VSCC	RR 10% OS 87%	RR 35% OS 42%	RR **p < 0.05** OS **p < 0.01**
Monk et al. 1995[17]	55	60%	40%	RR 27% OS 72%	RR 55% OS 44%	RR **p = 0.041** OS **p = 0.01**
Ansink et al. 1994[20]	60	32%	68%	RR NS OS NS	RR NS OS NS	RR NS OS HPV positive better OS; **p = 0.003**
Hording et al. 1993[24]	62	31%	69%	RR NS OS NS	RR NS OS NS	RR not significant OS NS
Bloss et al. 1991[18]	21	48%	52%	RR 20% OS 90%	RR 45% OS 82%	RR not significant OS not significant

*Determination of the presence of precursor lesions based on morphology.

HPV: human papilloma virus
VSCC: vulvar squamous cell carcinoma
RR: recurrence rate
OS: overall survival
NS: not specified

Table 4: Treatment of local recurrence of VSCC

Study	Total	Local	Local & groin	Beyond vulva*	Surgery	Surgery + RT	RT only	C-RT	No treatment/ palliative	Re-recurrences	5- year survival**	Note
Weikel, 2006[39]	N=201	Ns	ns	Ns	N=201 (100%)					68% after 5 years.	45%	Authors did not specify treatment for location of recurrent disease
Chakalova, 1993[94]	N=102	N=72 (70%)	N=18 (18%)	N=12 (12%)	N=81 (79%)	N=21 (21%)				NS	61%	79% of patients with a local recurrence were free of disease at last follow-up
Kohler, 1997[107]	N=82	N=39 (47%)	N=27 (33%)	N=16 (20%)	N=33 (40%)		N=16 (20%)		N=33 (40%)	17 (35%)	46.9%	Re-recurrence occurred after 12,8 months on average.
Piura, 1993[47]	N=73	N=39 (53%)		N=34 (47%)	N=41 (56%)	N=7 (10%)	N=5 (7%)		N=20 (27%)	NS	35.2%	At last follow up, 73% of all patients treated surgically were alive versus 25% of all patients treated with RT.
Schmidt, 1992[90]	N=51	N=28 (55%)	N=3 (6%)	N=20 (39%)	N=19 (37%)	N=20 (39%)	N=7 (14%)		N=5 (10%)	19 (37%)	61%	
Faul, 1998[84]	N=47	N=47 (100%)			N=31 (66%)	N=6 (13%)	N=6 (13%)	N=2 (4%)	N=2 (4%)	13 (28%) local, 6 (13%) groin or distant	40%	Mean time to re-recurrence 1,1 yr.
Frischbier, 1985[106]	N=41	N=41 (100%)			N=41 (100%)					NS	19.5%	All patients received radiotherapy as treatment for their primary tumor.
Strauβ, 1994[108]	N=37	N=28 (76%)	N=4 (11%)	N=5 (13%)	N=18 (49%)	N=8 (22%)	N=7 (19%)			50%	56%	For 4 patients treatment was NS

Study	Total	Local	Local & groin	Beyond vulva*	Surgery	Surgery + RT	RT only	C-RT	No treatment/ palliative	Re-recurrences	5- year survival**	Note
Hopkins, 1990[36]	N=34	N=24 (71%)	N=6 (17%)	N=4 (12%)	N=27 (79%)	N=7 (21%)				15 (44%)	61%	
Simonsen, 1984[91]	N=32	N=29 (91%)	N=3 (9%)		N=25 (78%)	N=5 (16%)	N=1 (3%)	N=1 (3%)		NS	NS	34% had no evidence of disease at last follow up.
Buchler, 1979[109]	N=21	N=18 (86%)	N=3 (14%)		N=13 (62%)	N=1 (5%)	N=7 (33%)			5 (38%) after S, 2 (29%) after RT	NS	Survival 43 months after S and 26 months after RT.
Raffetto, 2003[102]	N=20	N=6 (30%)	N=6 (30%)	N=8 (40%)			N=9 (45%)	N=11 (55%)		NS	20%	

Only studies that reported on a minimum of 20 patients were included
S: Surgery
RT: Radiotherapy
CT: Chemotherapy
C-RT: Chemoradiotherapy
NS: Not specified
CR: Complete response
PR: Partial response
*: Beyond vulva: all VSCC recurrences without a local component.
** For all patients: local, groin and distant recurrences
*** Radiotherapy sometimes combined with chemotherapy: not specified

3. Diagnosis and clinical evaluation

Symptoms of local recurrences differ, and patients may be asymptomatic. The diagnostic workup of patients with recurrent VSCC includes a complete medical history and full gynecological examination. All clinically suspect vulvar areas should be biopsied to confirm diagnosis and to glean information about the extent of disease. Fine-needle aspiration of suspected groin lymph nodes is needed to confirm the diagnosis (2, 4, 34, 96). For assessment of regional and distant metastases, computed tomography (CT) scans of the pelvis, abdomen, and chest are recommended (2, 4, 96). A positron emission tomography–CT scan may be considered for patients in whom other radiological imaging is inconclusive (2, 34, 96). If there is locally advanced recurrent VSCC, a cystoscopy and/or proctoscopy should be considered (40, 41).

4. Local recurrence

Surgery is the cornerstone of treatment for local recurrent VSCC (4, 8, 34, 96). Surgical treatment of local recurrences becomes more difficult with increasing tumor size, especially when the tumor is close to the anus or urethra. Furthermore, prior surgery with changed anatomy and, in particular, earlier radiotherapy can influence skin healing and the risk of wound dehiscence or infection. Reconstructive surgery can be an indispensable component of surgical treatment, for example by using local (fasciocutaneous) skin flaps (97) or V-Y reconstruction skin flaps from the upper posterior thighs (98-100) or split-skin grafts (101). Surgery may be contraindicated based on comorbidity and/or extensive previous surgery (1, 8, 34). In these cases, radiotherapy with or without concurrent chemotherapy may be considered, if not administered previously, preferably with the option of surgery for residual disease after downsizing (4, 102). Radiotherapy can be considered as an adjuvant or primary treatment (84). Chemotherapy is only indicated in combination with radiotherapy or as palliative treatment (102-104). A full IFL is considered standard treatment for the groins in cases of local recurrence infiltrating >1 mm, where primary treatment of VSCC did not comprise full IFL (8, 34, 36). A recent article on the safety of SLN biopsy in local recurrent VSCC showed that a repeat SLN biopsy is technically challenging, but feasible. The safety of the procedure should and will be further investigated before it is implemented in the treatment of local recurrent VSCC (105). We found 12 retrospective studies on the treatment of local recurrent VSCC. Table 4 provides an overview of these studies, with characteristics, reported number of re-recurrences, and 5-year overall survival (36, 39, 47, 84, 90, 91, 94, 102, 106-109).

4.1. Surgery

Patients who have an isolated local recurrence are good candidates for surgical treatment, unless there is a threat for the necessity of a colostomy. Eleven of the 12 studies on the treatment of local recurrent VSCC evaluated surgery alone as treatment for local recurrent VSCC. All 11 studies had a retrospective design. The largest study, published in 2006 by Weikel et al., included 201 patients (39). The other studies evaluated 13–81 patients. Surgery consisted of wide local excision, hemivulvectomy, or radical vulvectomy, with or without groin surgery. The type of surgery was based on the location and extent of the recurrence. The percentage of patients who developed a second recurrence was 28–50% (36, 39, 84, 90, 107-109), and 5-year survival was 20–79% (36, 39, 47, 84, 90, 94, 106-108). The most often encountered complications were wound infection (40%), vaginal stricture, and urinary incontinence (91, 109).

In seven studies, 5-21 patients with local recurrent VSCC were treated with surgery and adjuvant radiotherapy (36, 47, 84, 90, 91, 94, 108). The indications for adjuvant radiotherapy are unclear. In these studies, the percentage of subsequent recurrences was 35–50%, with a 5-year survival of 35–79%. Most reported complications after radiotherapy were skin reactions, such as moist desquamation and skin ulceration. In some cases, interruption of radiation treatment was necessary (102).

Pelvic exenteration may be a curative treatment option when patients have extensive locally recurrent VSCC that is otherwise untreatable. Four studies have reported on pelvic exenteration as a treatment option in cases of local recurrent VSCC. Pelvic exenteration achieved good symptom control, with a reported mean overall survival of 11 months and 2-year overall survival of 57%. This extensive surgical procedure is associated with considerable morbidity, and most patients develop psychological problems due to major alterations in body image and loss of sexual function. Patient selection and extensive counselling is of utmost importance before pelvic exenteration is performed (110-113).

4.2 (Chemo)radiotherapy

When surgery is not possible or may lead to high morbidity, (chemo)radiotherapy can be considered as a primary treatment for locally recurrent VSCC, but only if patients have not previously undergone radiotherapy. Therapy plans are individualized depending on the extent of disease and prior therapy, but they most often involve external beam radiotherapy (EBRT), in some cases with a brachytherapy boost. Seven retrospective studies were performed that included 5–20 patients treated with primary (chemo) radiotherapy for locally recurrent VSCC (47, 84, 90, 102, 107-109). In general, treatment with primary (chemo)radiotherapy yields less favorable treatment results than surgery with respect to 5-year survival (20–60% versus 20–79%, respectively) (47, 109). It should, however, be emphasised that there is a high risk of bias with regard to therapy

selection and associated outcomes. Although most studies do not report on the selection of therapy, it is plausible that patients with worse clinical or tumor characteristics were selected more often for (chemo)radiotherapy instead of surgical therapy. Radiotherapy-associated side effects were severe skin desquamation (20%) (102), radiation fibrosis (10%) (102), lymphedema (10%) (102), and, more rarely, radiation proctitis (3%). The additional value of concurrent chemoradiotherapy in recurrent VSCC is not well documented. However, this treatment strategy has been suggested to improve salvage of bulky locally advanced disease, with complete response rates as high as 64% (102, 114-116). One study evaluated the efficacy and toxicity of chemotherapy alone (cisplatin and vinorelbine); it included nine patients with local recurrent VSCC and seven patients with a groin recurrence. A complete response was recorded in 27% of the patients and a partial response in 13%. Stable disease was observed in 27% of the patients and progressive disease in 33%. The median progression-free survival was 10 months (range 3–17 months), and overall survival was 19 months (1–30 months). Toxicity of the treatment was high, especially hematological toxicity; 31% of the patients experienced WHO grade 3/4 leukopenia, 69% had neutropenia, and 24% had anemia. Other WHO grade 3/4 toxicities included nausea/vomiting in 62% of the patients, neurotoxicity in 38%, and alopecia in 62% (117). Chemotherapy as treatment for locally recurrent VSCC should only be considered as a last resort in a palliative treatment setting, with a small chance of response.

5. Groin recurrence

Nearly all VSCC patients with a groin recurrence die of disease, and management is challenging (4, 6, 34, 38, 50, 51, 64-66). However, a recently published study on groin recurrences found a 50% survival rate after 7 years and the authors suggest that a groin recurrence should therefore no longer be considered a palliative situation (95). Choice of treatment is individualized and determined by the size of the tumor, previous treatment, and time interval to recurrence (34, 38, 64, 66). We found a total of 7 studies that reported on the treatment of groin recurrences in VSCC patients. All studies had a retrospective design (35, 36, 85, 91-93, 95). Patients included in the studies had local and groin recurrences, isolated groin recurrences or groin and pelvic recurrence. An overview of the studies and their results is provided in table 5. Median survival was 3–19 months, with overall survival rates of 0–50%.

Surgery, followed by radiotherapy when possible, is currently the treatment of choice if the patient is in good general health. Primary radiotherapy for a groin recurrence can be considered as alternative treatment, but almost never leads to a cure (35, 118, 119). Five studies report on surgery, either alone or in combination with radiotherapy, as a treatment for groin recurrences. Surgical treatment consisted of full IFL or debulking of the groin recurrence(s). Radiotherapy consisted of external beam therapy. Some patients in these studies received primary radiotherapy. In general, the recommended dose for radiotherapy is 46–50 Gy in fractions of 1.8–2.0 Gy, with a boost to 56–60 Gy to the site of the involved lymph node(s), especially in cases of extracapsular extension, and to 64–66 Gy to macroscopic residual disease. One study combined surgery with chemo- and radiotherapy in 10 of the 30 included patients. Which chemotherapy was not specified by the authors (95). Progression-free survival and overall survival was low, with median survival rates varying from 6 to 16 months. Only a few patients survived for >5 years without evidence of disease after treatment (35, 36, 85, 91). However, in the most recently published study a five-year survival of 50% was found. Especially patients who underwent combined therapy, surgery with (chemo)radiotherapy had a better overall survival after groin recurrence in comparison to patients with single-mode therapy (HR 0.25, p=0.037)(95). Earlier studies already suggested better outcomes for concurrent chemoradiotherapy, also based on efficacy of the treatment for advanced primary disease (115, 120). Concurrent chemoradiotherapy is recommended for treatment of (bulky) groin recurrence, followed by resection of the residual tumor if feasible (120-122). Chemotherapy as a stand-alone treatment for groin recurrence is only considered in a palliative setting, when surgery or radiotherapy are not advisable (92, 93). Two studies evaluated palliative treatment of a groin recurrence with chemotherapy alone. These studies found a median progression-free survival of 2.6–4 months, with a median overall survival of 7–9 months (92, 93). In other words, palliative chemotherapy for groin recurrences yields short response rates with substantial side effects.

5.1 Skin bridge recurrence

Skin bridge recurrence can be considered a special type of locoregional recurrence, with a clinical course comparable to groin recurrence and an extremely poor prognosis, despite treatment with surgery and/or radiotherapy. It has been hypothesized that skin bridge recurrences evolve from metastatic tumor emboli in lymphatic vessels arrested in their migration (9). Skin bridge recurrences in vulvar cancer are uncommon, but they are relevant because of the poor outcome. The incidence has been described as 0–9% (9, 36, 46, 47, 50, 123). Since the introduction of a surgical approach with separate incisions (instead of “en-bloc” surgery), there has been an increased incidence of skin bridge recurrences (46). De Hullu et al. investigated the prevalence of groin and skin bridge recurrences in 253 VSCC patients primarily treated with surgery. Group I underwent radical vulvectomy with en bloc IFL, and group II underwent wide local excision with

Table 5: Treatment of groin recurrence of VSCC

Study	Total	Local & groin	Isolated groin	Surgery	Surgery + RT	RT	CT	Response	Progression-free survival	Overall survival	Note
Frey, 2016[95]	N=30		N=30* (7 patients had groin and pelvic recurrence)	N=7 (23%)	N=20 (66%), 10 patients received surgery and C-RT	N=1 (3%)	N=1 (3%)	NS	NS	50% 5-year survival	Patients with multimodal groin treatments performed better than tose with single-mode treatment (hazard ratio 0.25, p=0.037) * No data available of 1 patient
Cormio, 2010[35]	N=21	N=4 (21%), 2 local, groin & distant recurrence	N=17 (81%)	N=3 (14%)	N=7 (34%)	N=2 (10%)	N=3 (14%)	NS	NS	Median: 9 months (range 3-30)	95% of the patients died, 1 patient alive after 60 months after surgical treatment. 3 (14%) patients received surgery and CT and 3 (14%) patients refused treatment.
Hopkins, 1990[36]	N=10	N=6 (60%)	N=4 (40%)	N=4 (40%)	N=6 (60%)			NS	0/10	10% 5-year survival	
Simonsen, 1984[91]	N=12	N=3 (25%)	N=9 (75%)	N=1 (8%)	N=4 (34%)	N=7 (58%)		NS	NS	Median: 6 months (0.1-3.5 years). 8,3% 5-year survival	1 patient had NED after 6 years. She was treated with surgery and RT.
Tilmans, 1992[85]	N=12		N=12 (100%)		N=5 (42%)	N=5 (42%)	N=1 (8%)	NS	17%	Median: 10 months	2 patients received cyclophosphamide & cisplatin after S+RT. 1 patient received supportive care
Wagenaar, 2001[92]	N=13		N=13 (100%)				N=13 (100%)	PR: 54%, 7/13	Median: 4 months (range 2-22)	Median: 9 months (range 2-31)	CT: bleomycin, methotrexate & CCNU.
Study	**Total**	**Local & groin**	**Isolated groin**	**Surgery**	**Surgery + RT**	**RT**	**CT**	**Response**	**Progression-free survival**	**Overall survival**	**Note**

Witteveen, 2009[93]	N=11	N=5 (45%)	N=6 (55%)	N=11 (100%)	CR + PR 27%, 3/11	Median: 2,6 months (2-4,2 months). 10,3% 1-year progression free-survival	Median: 6,9 months (range 3,5-12,4). 31% 1-year OS	Patients were not amenable to surgery or radiotherapy. Response and overall survival is for all patients included in the study (total 29, all patients with locally advanced or recurrent VSCC) CT: paclitaxel.

Only studies that reported on a minimum of 10 patients were included

S: Surgery
RT: Radiotherapy
CT: Chemotherapy
C-RT: Chemoradiotherapy
CT: Chemotherapy
NS: Not specified
CR: Complete response
PR: Partial response
SD: Stable disease
PD: Progressive disease
NED: no evidence of disease

IFL through separate incisions. The prevalence of skin bridge recurrences was 6.3% in group II compared to 1.3% in group I (p=0.029)(46). Rose et al. found five skin bridge recurrences in a group of 126 patients (3.9%) with VSCC (123). Rouzier et al. investigated relapses and prognostic factors associated with skin bridge recurrences. They found that margin status (p=0.001) and tumor size >2 cm (p<0.05) were significantly associated with the occurrence of skin bridge recurrences. Seven patients had a skin bridge recurrence out of a group of 215 VSCC patients. None of the seven patients were alive after 1 year (9). On the other hand, Woolderink et al. reported on 125 patients with VSCC; none had a skin bridge recurrence (50). In conclusion, literature on skin bridge recurrences is limited and prognosis is still very poor.

6. Distant recurrence

Patients with distant recurrence have a very poor prognosis. There is no standard therapy for these patients, and treatment is always palliative (85, 96). In cases of isolated recurrence in the pelvis, radiotherapy or concurrent chemoradiotherapy can be considered. Patients with para-aortic nodal recurrences can be treated with radiation therapy to relieve symptoms. Radiation can also be used for palliation of pain due to bone metastasis. Some studies have evaluated chemotherapy for patients with metastatic vulvar cancer, often as a last resort if patients are not amenable to surgery or radiotherapy. These studies are summarized in table 6 (85, 93, 102, 124). The most commonly used agents are paclitaxel, bleomycin, cisplatin and 5-fluorouracil, but large series are lacking. Although some regimens were associated with limited clinical activity, response rates were low, with complete response rates of 7–20% (93, 102) and partial response rates of 7–80% (93, 102, 124). Other patients had stable or progressive disease during chemotherapy (85, 93, 124). Response was usually short, with a median survival of 4–7 months. Response rates of recurrences in irradiated areas are even lower.

Recently, EGFR targeting therapy has been suggested as a therapeutic option in the treatment of patients with distant recurrences. In a phase II trial on the effect of erlotinib (an inhibitor of the EGFR tyrosine kinase) 32 patients with distant recurrences were included. Eight patients were treated with one cycle of 28 days oral erlotinib followed by surgery or chemoradiation (cohort 1) and 24 patients were treated with multiple cycles of oral erlotinib (cohort 2, mean 3.3 cycles). In cohort 1 35% of the patients showed a partial response to erlotinib therapy and in cohort 2 22% of the patients. Progressive disease was seen in 6% and 23% of the patients, respectively. Other patients had stable disease or were unevaluable, because they failed to complete a minimum number of cycles of therapy due to serious adverse events. Adverse events included an allergic reaction, diarrhoea, dehydration, electrolyte abnormalities, gastro-intestinal bleed and ischemic colitis (all grade 3 toxicity). Two patients experienced grade 4 acute renal

Table 6: Chemotherapy for patients with distant recurrent VSCC

Study	N° patients distant recurrent disease	CT regimen	Response	Survival	Note
Witteveen, 2009[93]	8 distant (22 total)	Paclitaxel	CR 7% PR 7% Overall response 14%	Median 6.9 months 1-year survival 31%	4 patients discontinued treatment for toxicity
Raffetto, 2003[102]	7 distant	Cisplatin or Cisplatin with 5 FU	CR 20% (1/5) PR 80% (4/5)	Median; 6 months (range 2-16). Mean; 8,2 months.	All patients also got radiotherapy. Five patients were treated with chemotherapy concomitantly or neo-adjuvant.
Tilmans, 1992[85]	11 distant	5 FU, Cisplatin, Aziridinyl-benzequinone, etoposide, doxorubicin, cyclophosphamide	PD 100%	Median; 4 months pelvic recurrence and 5 months distant recurrence	All treated with a palliative intention
Durrant, 1990[124]	10 distant	Bleomycin, methotrexate and iomustine	6/10 (60%) showed a partial or complete response	ns	Administered to patients ineligible for surgical treatment. 33 % of the patients discontinued treatment due to severe toxicity.

CT: Chemotherapy
OS: Overall Survival
CR: Complete Response
PR: Partial Response
SD: Stable disease
PD: Progressive disease
NS: not specified

failure (125). These results are promising and new studies regarding targeted therapies in VSCC patients with distant recurrences are expected in the next future.

7. Summary and recommendations

In cases of local recurrence, surgical resection is the treatment of choice. If needed, combined with reconstructive and/or groin surgery. If primary surgery is not an option because of tumor growth adjacent to the urethra or anus, (chemo)radiotherapy can be a good alternative, either as definitive treatment or prior to surgery for downsizing the tumor. Chemotherapy alone is considered palliative treatment and is not recommended due to the low response rates and short duration of response. The preferred treatment for groin and skin bridge recurrence is surgery, followed by radiation therapy if not previously irradiated. Concurrent chemoradiotherapy can be considered if primary resection does not seem feasible, either preoperatively for downsizing of a bulky groin recurrence or as definitive or palliative treatment. Distant recurrences of VSCC are rare, and treatment is only palliative. Chemotherapy can be considered, but it has low response rates. Future studies regarding targeted treatment in patients with metastatic VSCC are expected. Management of recurrent VSCC should be individualized and requires an experienced, multidisciplinary team approach in an oncological center.

References

1. Gadducci A, Cionini L, Romanini A, Fanucchi A, Genazzani AR. Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer. Crit RevOncolHematol. 2006;60(3):227-41.
2. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.
3. de Hullu JA, van der Zee AG. Surgery and radiotherapy in vulvar cancer. Crit RevOncolHematol. 2006;60(1):38-58.
4. Berek JS, Hacker N.F. Berek and Hacker's Gynecologic Oncology. 2014;6 ed Philadelphia LWW.
5. Schuurman MS, van den Einden LC, Massuger LF, Kiemeney LA, van der Aa MA, de Hullu JA. Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma. EurJCancer. 2013;49(18):3872-80.
6. Gonzalez BJ, Magrina JF, Gaffey TA, Hernandez JL, Webb MJ, Cliby WA, et al. Long-term survival and disease recurrence in patients with primary squamous cell carcinoma of the vulva. GynecolOncol. 2005;97(3):828-33.
7. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
8. Salom EM, Penalver M. Recurrent vulvar cancer. Curr TreatOptions Oncol. 2002;3(2):143-53.
9. Rouzier R, Haddad B, Plantier F, Dubois P, Pelisse M, Paniel BJ. Local relapse in patients treated for squamous cell vulvar carcinoma: incidence and prognostic value. Obstet Gynecol. 2002;100(6):1159-67.
10. Oncology FCoG. FIGO staging for carcinoma of the vulva, cervix, and corpus uteri. Int J Gynaecol Obstet. 2014;125(2):97-8.
11. van der Steen S, de Nieuwenhof HP, Massuger L, Bulten J, de Hullu JA. New FIGO staging system of vulvar cancer indeed provides a better reflection of prognosis. GynecolOncol. 2010;119(3):520-5.
12. Al-Ghamdi A, Freedman D, Miller D, Poh C, Rosin M, Zhang L, et al. Vulvar squamous cell carcinoma in young women: a clinicopathologic study of 21 cases. GynecolOncol. 2002;84(1):94-101.
13. Alonso I, Fuste V, Del PM, Castillo P, Torne A, Fuste P, et al. Does human papillomavirus infection imply a different prognosis in vulvar squamous cell carcinoma? GynecolOncol. 2011;122(3):509-14.
14. Canavan TP, Cohen D. Vulvar cancer. AmFamPhysician. 2002;66(7):1269-74.
15. Kokka F, Singh N, Faruqi A, Gibbon K, Rosenthal AN. Is differentiated vulval intraepithelial neoplasia the precursor lesion of human papillomavirus-negative vulval squamous cell carcinoma? IntJGynecolCancer. 2011;21(7):1297-305.
16. Lindell G, Nasman A, Jonsson C, Ehrsson RJ, Jacobsson H, Danielsson KG, et al. Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node. GynecolOncol. 2010;117(2):312-6.
17. Monk BJ, Burger RA, Lin F, Parham G, Vasilev SA, Wilczynski SP. Prognostic significance of human papillomavirus DNA in vulvar carcinoma. ObstetGynecol. 1995;85(5 Pt 1):709-15.
18. Bloss JD, Liao SY, Wilczynski SP, Macri C, Walker J, Peake M, et al. Clinical and histologic features of vulvar carcinomas analyzed for human papillomavirus status: evidence that squamous cell carcinoma of the vulva has more than one etiology. HumPathol. 1991;22(7):711-8.

19. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. Crit RevOncolHematol. 2008;68(2):131-56.
20. Ansink AC, Krul MR, De Weger RA, Kleyne JA, Pijpers H, Van TH, et al. Human papillomavirus, lichen sclerosus, and squamous cell carcinoma of the vulva: detection and prognostic significance. GynecolOncol. 1994;52(2):180-4.
21. Coleman RL, Santoso JT. Vulvar carcinoma. CurrTreatOptionsOncol. 2000;1(2):177-90.
22. Larsson GL, Helenius G, Andersson S, Elgh F, Sorbe B, Karlsson MG. Human papillomavirus (HPV) and HPV 16-variant distribution in vulvar squamous cell carcinoma in Sweden. Int J Gynecol Cancer. 2012;22(8):1413-9.
23. Pinto AP, Schlecht NF, Pintos J, Kaiano J, Franco EL, Crum CP, et al. Prognostic significance of lymph node variables and human papillomavirus DNA in invasive vulvar carcinoma. Gynecol Oncol. 2004;92(3):856-65.
24. Hording U, Kringsholm B, Andreasson B, Visfeldt J, Daugaard S, Bock JE. Human papillomavirus in vulvar squamous-cell carcinoma and in normal vulvar tissues: a search for a possible impact of HPV on vulvar cancer prognosis. IntJCancer. 1993;55(3):394-6.
25. van de Nieuwenhof HP, Massuger LF, van der Avoort IA, Bekkers RL, Casparie M, Abma W, et al. Vulvar squamous cell carcinoma development after diagnosis of VIN increases with age. EurJCancer. 2009;45(5):851-6.
26. Abell MR, Gosling JR. Intraepithelial and infiltrative carcinoma of vulva: Bowen's type. Cancer. 1961;14:318-29.
27. Bornstein J. The 2015 Internation Society for the Study of Vulvovaginal Disease Terminology of Vulvar Squamous Intraepithelial Lesions. 2015.
28. Torezan LA, Festa-Neto C. Cutaneous field cancerization: clinical, histopathological and therapeutic aspects. AnBrasDermatol. 2013;88(5):775-86.
29. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res. 2003;63(8):1727-30.
30. van der Zee AG, Oonk MH, de Hullu JA, Ansink AC, Vergote I, Verheijen RH, et al. Sentinel node dissection is safe in the treatment of early-stage vulvar cancer. JClinOncol. 2008;26(6):884-9.
31. Oonk MH, van Hemel BM, Hollema H, de Hullu JA, Ansink AC, Vergote I, et al. Size of sentinel-node metastasis and chances of non-sentinel-node involvement and survival in early stage vulvar cancer: results from GROINSS-V, a multicentre observational study. Lancet Oncol. 2010;11(7):646-52.
32. Oonk MH, de Hullu JA, van der Zee AG. Current controversies in the management of patients with early-stage vulvar cancer. CurrOpinOncol. 2010;22(5):481-6.
33. van den Einden LC, Aben KK, Massuger LF, van Spronsen DJ, de Hullu JA. Successful centralisation of patients with vulvar carcinoma: a population-based study in The Netherlands. EurJCancer. 2012;48(13):1997-2003.
34. Coulter J, Gleeson N. Local and regional recurrence of vulval cancer: management dilemmas. Best Pract Res Clin Obstet Gynaecol. 2003;17(4):663-81.
35. Cormio G, Loizzi V, Carriero C, Cazzolla A, Putignano G, Selvaggi L. Groin recurrence in carcinoma of the vulva: management and outcome. EurJCancer Care (Engl). 2010;19(3):302-7.

36. Hopkins MP, Reid GC, Morley GW. The surgical management of recurrent squamous cell carcinoma of the vulva. Obstet Gynecol. 1990;75(6):1001-5.
37. Oonk MH, Hollema H, de Hullu JA, van der Zee AG. Prediction of lymph node metastases in vulvar cancer: a review. IntJGynecolCancer. 2006;16(3):963-71.
38. Stehman FB, Bundy BN, Ball H, Clarke-Pearson DL. Sites of failure and times to failure in carcinoma of the vulva treated conservatively: a Gynecologic Oncology Group study. AmJObstetGynecol. 1996;174(4):1128-32.
39. Weikel W, Schmidt M, Steiner E, Knapstein PG, Koelbl H. Surgical therapy of recurrent vulvar cancer. Am J Obstet Gynecol. 2006;195(5):1293-302.
40. Guideline vulvar cancer, national cancer institute. 2015.
41. Guideline vulvar cancer, oncoline. 2011.
42. Guidelines for the Diagnosis and Management of Vulval Carcinoma, RCOG. 2014.
43. N.F. H, J.C. G, C.J. H. Essentials of Obstetrics and Gynecology. Philadelphia: Elsevier inc.; 2010 2010.
44. A A, M G, P D. Textbook of gynaecological oncology. 2nd ed ed. Ankara: Günes Publishing; 2010 8/2010.
45. Maggino T, Landoni F, Sartori E, Zola P, Gadducci A, Alessi C, et al. Patterns of recurrence in patients with squamous cell carcinoma of the vulva. A multicenter CTF Study. Cancer. 2000;89(1):116-22.
46. de Hullu JA, Hollema H, Lolkema S, Boezen M, Boonstra H, Burger MP, et al. Vulvar carcinoma. The price of less radical surgery. Cancer. 2002;95(11):2331-8.
47. Piura B, Masotina A, Murdoch J, Lopes A, Morgan P, Monaghan J. Recurrent squamous cell carcinoma of the vulva: a study of 73 cases. Gynecol Oncol. 1993;48(2):189-95.
48. Te Grootenhuis NC, van der Zee AG, van Doorn HC, van d, V, Vergote I, Zanagnolo V, et al. Sentinel nodes in vulvar cancer: Long-term follow-up of the GROningen INternational Study on Sentinel nodes in Vulvar cancer (GROINSS-V) I. GynecolOncol. 2016;140(1):8-14.
49. Oonk MH, de Hullu JA, Hollema H, Mourits MJ, Pras E, Wymenga AN, et al. The value of routine follow-up in patients treated for carcinoma of the vulva. Cancer. 2003;98(12):2624-9.
50. Woolderink JM, de Bock GH, de Hullu JA, Davy MJ, van der Zee AG, Mourits MJ. Patterns and frequency of recurrences of squamous cell carcinoma of the vulva. GynecolOncol. 2006;103(1):293-9.
51. Woelber L, Mahner S, Voelker K, Eulenburg CZ, Gieseking F, Choschzick M, et al. Clinicopathological prognostic factors and patterns of recurrence in vulvar cancer. Anticancer Res. 2009;29(2):545-52.
52. Preti M, Ronco G, Ghiringhello B, Micheletti L. Recurrent squamous cell carcinoma of the vulva: clinicopathologic determinants identifying low risk patients. Cancer. 2000;88(8):1869-76.
53. Rutledge FN, Mitchell MF, Munsell MF, Atkinson EN, Bass S, McGuffee V, et al. Prognostic indicators for invasive carcinoma of the vulva. Gynecol Oncol. 1991;42(3):239-44.
54. Bogani G, Cromi A, Serati M, Uccella S, Donato VD, Casarin J, et al. Predictors and Patterns of Local, Regional, and Distant Failure in Squamous Cell Carcinoma of the Vulva. AmJClinOncol. 2014.
55. Yoder BJ, Rufforny I, Massoll NA, Wilkinson EJ. Stage IA vulvar squamous cell carcinoma: an analysis of tumor invasive characteristics and risk. Am J Surg Pathol. 2008;32(5):765-72.

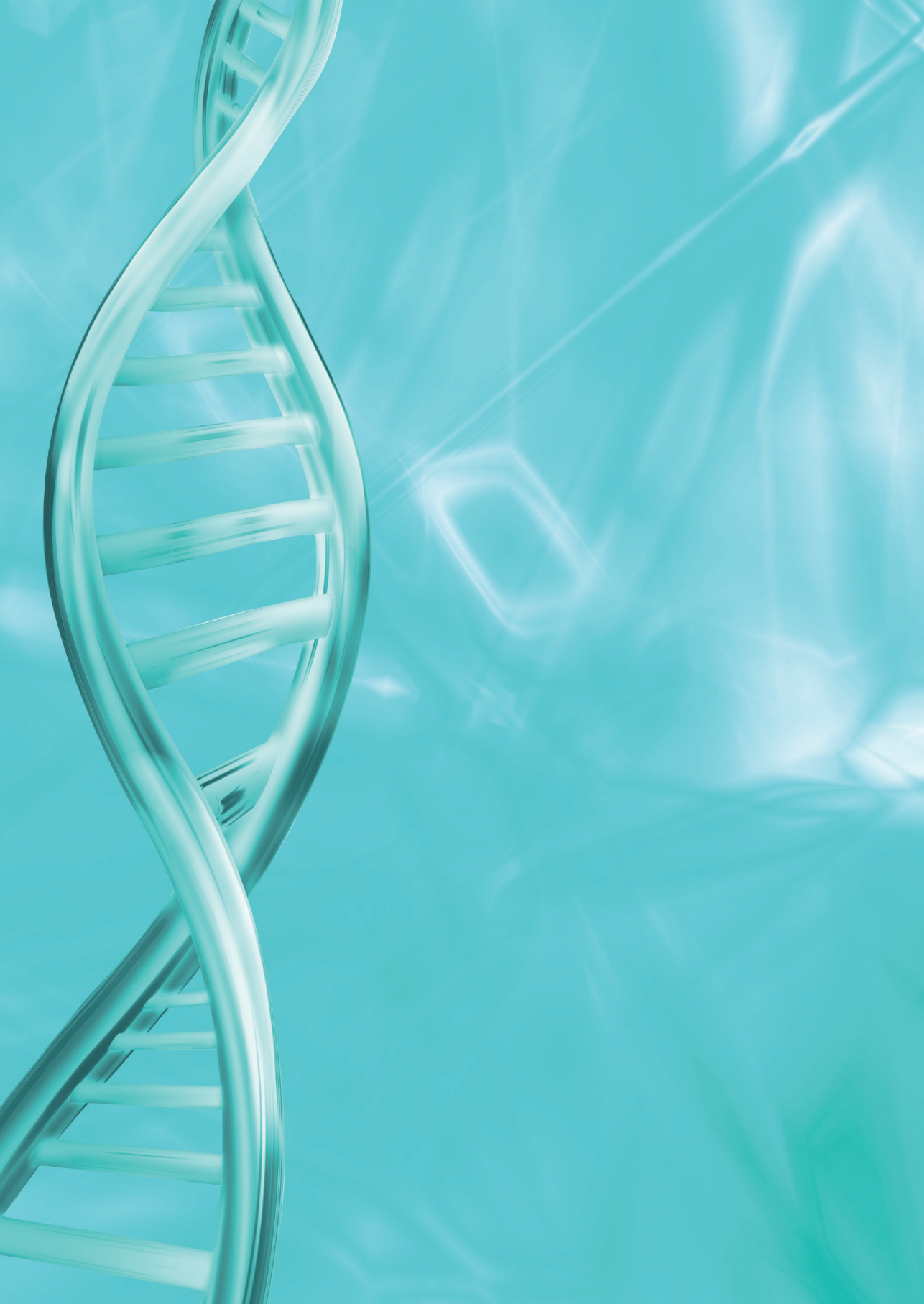
56. Holthoff ER, Jeffus SK, Gehlot A, Stone R, Erickson SW, Kelly T, et al. Perineural Invasion Is an Independent Pathologic Indicator of Recurrence in Vulvar Squamous Cell Carcinoma. Am J Surg Pathol. 2015;39(8):1070-4.
57. Regauer S. Residual anogenital lichen sclerosus after cancer surgery has a high risk for recurrence: a clinicopathological study of 75 women. GynecolOncol. 2011;123(2):289-94.
58. Modesitt SC, Waters AB, Walton L, Fowler WC, Jr., Van LL. Vulvar intraepithelial neoplasia III: occult cancer and the impact of margin status on recurrence. Obstet Gynecol. 1998;92(6):962-6.
59. Heaps JM, Fu YS, Montz FJ, Hacker NF, Berek JS. Surgical-pathologic variables predictive of local recurrence in squamous cell carcinoma of the vulva. Gynecol Oncol. 1990;38(3):309-14.
60. Viswanathan AN, Pinto AP, Schultz D, Berkowitz R, Crum CP. Relationship of margin status and radiation dose to recurrence in post-operative vulvar carcinoma. Gynecol Oncol. 2013;130(3):545-9.
61. Groenen SM, Timmers PJ, Burger CW. Recurrence rate in vulvar carcinoma in relation to pathological margin distance. Int J Gynecol Cancer. 2010;20(5):869-73.
62. Dakubo GD, Jakupciak JP, Birch-Machin MA, Parr RL. Clinical implications and utility of field cancerization. Cancer Cell International. 2007;7 :15 Mar 2007.
63. Woelber L, Choschzick M, Eulenburg C, Hager M, Jaenicke F, Gieseking F, et al. Prognostic value of pathological resection margin distance in squamous cell cancer of the vulva. Ann Surg Oncol. 2011;18(13):3811-8.
64. Deka P, Barmon D, Shribastava S, Kataki AC, Sharma JD, Bhattacharyya M. Prognosis of vulval cancer with lymph node status and size of primary lesion: A survival study. JMidlifeHealth. 2014;5(1):10-3.
65. Woelber L, Eulenburg C, Choschzick M, Kruell A, Petersen C, Gieseking F, et al. Prognostic role of lymph node metastases in vulvar cancer and implications for adjuvant treatment. IntJGynecolCancer. 2012;22(3):503-8.
66. Lataifeh I, Nascimento MC, Nicklin JL, Perrin LC, Crandon AJ, Obermair A. Patterns of recurrence and disease-free survival in advanced squamous cell carcinoma of the vulva. GynecolOncol. 2004;95(3):701-5.
67. Mahner S, Jueckstock J, Hilpert F, Neuser P, Harter P, de Gregorio N, et al. Adjuvant therapy in lymph node-positive vulvar cancer: the AGO-CaRE-1 study. Journal of the National Cancer Institute. 2015;107(3).
68. Homesley HD, Bundy BN, Sedlis A, Adcock L. Radiation therapy versus pelvic node resection for carcinoma of the vulva with positive groin nodes. Obstet Gynecol. 1986;68(6):733-40.
69. Kunos C, Simpkins F, Gibbons H, Tian C, Homesley H. Radiation therapy compared with pelvic node resection for node-positive vulvar cancer: a randomized controlled trial. ObstetGynecol. 2009;114(3):537-46.
70. Hacker NF, Berek JS, Lagasse LD, Leuchter RS, Moore JG. Management of regional lymph nodes and their prognostic influence in vulvar cancer. ObstetGynecol. 1983;61(4):408-12.
71. Aragona AM, Cuneo NA, Soderini AH, Alcoba EB. An analysis of reported independent prognostic factors for survival in squamous cell carcinoma of the vulva: is tumor size significance being underrated? GynecolOncol. 2014;132(3):643-8.

72. Courtney-Brooks M, Sukumvanich P, Beriwal S, Zorn KK, Richard SD, Krivak TC. Does the number of nodes removed impact survival in vulvar cancer patients with node-negative disease? GynecolOncol. 2010;117(2):308-11.
73. van Beekhuizen HJ, Auzin M, van den Einden LC, de Hullu JA, van d, V, Wildhagen MF, et al. Lymph node count at inguinofemoral lymphadenectomy and groin recurrences in vulvar cancer. IntJGynecolCancer. 2014;24(4):773-8.
74. Baiocchi G, Cestari FM, Rocha RM, Faloppa CC, Kumagai LY, Fukazawa EM, et al. Does the count after inguinofemoral lymphadenectomy in vulvar cancer correlate with outcome? Eur J Surg Oncol. 2013;39(4):339-43.
75. Chen SL, Iddings DM, Scheri RP, Bilchik AJ. Lymphatic mapping and sentinel node analysis: current concepts and applications. CA Cancer JClin. 2006;56(5):292-309.
76. Paladini D, Cross P, Lopes A, Monaghan JM. Prognostic significance of lymph node variables in squamous cell carcinoma of the vulva. Cancer. 1994;74(9):2491-6.
77. Ansink A, van d, V. Surgical interventions for early squamous cell carcinoma of the vulva. Cochrane Database Syst Rev. 2000(2):CD002036.
78. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. ClinCancer Res. 2009;15(22):6758-62.
79. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. JNatlCancer Inst. 2008;100(4):261-9.
80. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. IntJCancer. 2007;121(8):1813-20.
81. de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. The Lancet Oncology. 2012;13(6):607-15.
82. Mensah FA, Mehta MR, Lewis JS, Jr., Lockhart AC. The Human Papillomavirus Vaccine: Current Perspective and Future Role in Prevention and Treatment of Anal Intraepithelial Neoplasia and Anal Cancer. The oncologist. 2016.
83. Rouzier R, Morice P, Haie-Meder C, Lhomme C, Avril MF, Duvillard P, et al. Prognostic significance of epithelial disorders adjacent to invasive vulvar carcinomas. GynecolOncol. 2001;81(3):414-9.
84. Faul C, Miramow D, Gerszten K, Huang C, Edwards R. Isolated local recurrence in carcinoma of the vulva: Prognosis and implications for treatment. International Journal of Gynecological Cancer. 1998;8(5):409-14.
85. Tilmans AS, Sutton GP, Look KY, Stehman FB, Ehrlich CE, Hornback NB. Recurrent squamous carcinoma of the vulva. Am J Obstet Gynecol. 1992;167(5):1383-9.
86. Onnis A, Marchetti M, Maggino T. Carcinoma of the vulva: critical analysis of survival and treatment of recurrences. Eur J Gynaecol Oncol. 1992;13(6):480-5.
87. Trietsch MD, Peters AA, Gaarenstroom KN, van Koningsbrugge SH, ter Haar NT, Osse EM, et al. Spindle cell morphology is related to poor prognosis in vulvar squamous cell carcinoma. BrJCancer. 2013;109(8):2259-65.

88. Trietsch MD, Spaans VM, ter Haar NT, Osse EM, Peters AA, Gaarenstroom KN, et al. CDKN2A(p16) and HRAS are frequently mutated in vulvar squamous cell carcinoma. GynecolOncol. 2014;135(1):149-55.
89. Drew PA, al-Abbadi MA, Orlando CA, Hendricks JB, Kubilis PS, Wilkinson EJ. Prognostic factors in carcinoma of the vulva: a clinicopathologic and DNA flow cytometric study. IntJGynecolPathol. 1996;15(3):235-41.
90. Schmidt W, Villena-Heinsen C, Schmid H, Engel K, Jochum N, Muller A. [Recurrence of vulvar cancer--treatment, experiences and results]. Geburtshilfe Frauenheilkd. 1992;52(8):462-6.
91. Simonsen E. Treatment of recurrent squamous cell carcinoma of the vulva. Acta Radiol Oncol. 1984;23(5):345-8.
92. Wagenaar HC, Colombo N, Vergote I, Hoctin-Boes G, Zanetta G, Pecorelli S, et al. Bleomycin, methotrexate, and CCNU in locally advanced or recurrent, inoperable, squamous-cell carcinoma of the vulva: an EORTC Gynaecological Cancer Cooperative Group Study. European Organization for Research and Treatment of Cancer. Gynecol Oncol. 2001;81(3):348-54.
93. Witteveen PO, van d, V, Vergote I, Guerra C, Scarabeli C, Coens C, et al. Phase II study on paclitaxel in patients with recurrent, metastatic or locally advanced vulvar cancer not amenable to surgery or radiotherapy: a study of the EORTC-GCG (European Organisation for Research and Treatment of Cancer--Gynaecological Cancer Group). Ann Oncol. 2009;20(9):1511-6.
94. Chakalova G, Karagiozov A. The surgical management of recurrent carcinoma of the vulva. Eur J Gynaecol Oncol. 1993;14(4):318-22.
95. Frey JN, Hampl M, Mueller MD, Gunthert AR. Should Groin Recurrence Still Be Considered as a Palliative Situation in Vulvar Cancer Patients?: A Brief Report. Int J Gynecol Cancer. 2016;26(3):575-9.
96. Fonseca-Moutinho JA. Recurrent vulvar cancer. ClinObstetGynecol. 2005;48(4):879-83.
97. Weikel W, Hofmann M, Steiner E, Knapstein PG, Koelbl H. Reconstructive surgery following resection of primary vulvar cancers. GynecolOncol. 2005;99(1):92-100.
98. Lee PK, Choi MS, Ahn ST, Oh DY, Rhie JW, Han KT. Gluteal fold V-Y advancement flap for vulvar and vaginal reconstruction: a new flap. PlastReconstrSurg. 2006;118(2):401-6.
99. Saito A, Minakawa H, Saito N, Isu K, Hiraga H, Osanai T. Posterior thigh flap revisited: clinical use in oncology patients. SurgToday. 2014;44(6):1013-7.
100. Rinaldi M, Cormio G, Bucaria V, Di TP, Marino F, Altomare DF. [Reconstruction with skin flaps of the posterior aspect of the thighs after total pelvic evisceration with removal of vulvo-perineal soft tissues in recurrent vulvar squamous carcinoma]. Suppl Tumori. 2005;4(3):S208.
101. Thomas SS, Chenoy R, Fielding JW, Rollason TP, Jordan JA, Bracka A. Vulvoperineal reconstruction after excision of anogenital multifocal intraepithelial neoplasia ("MIN"). BrJPlastSurg. 1996;49(8):539-46.
102. Raffetto N, Tombolini V, Santarelli M, Valeriani M, Galla DA, Enrici RM. Radiotherapy alone and chemoirradiation in recurrent squamous cell carcinoma of the vulva. Anticancer Res. 2003;23(3C):3105-8.
103. Whitaker SJ, Kirkbride P, Arnott SJ, Hudson CN, Shepherd JH. A pilot study of chemo-radiotherapy in advanced carcinoma of the vulva. BrJObstetGynaecol. 1990;97(5):436-42.

104. Russell AH, Mesic JB, Scudder SA, Rosenberg PJ, Smith LH, Kinney WK, et al. Synchronous radiation and cytotoxic chemotherapy for locally advanced or recurrent squamous cancer of the vulva. Gynecol Oncol. 1992;47(1):14-20.
105. Doorn HC, van Beekhuizen HJ, Gaarenstroom KN, van d, V, van der Zee AG, Oonk M, et al. Repeat sentinel lymph node procedure in patients with recurrent vulvar squamous cell carcinoma is feasible. GynecolOncol. 2016.
106. Frischbier HJ, Wenn K. [Secondary vulvectomy treatment results in local recurrence of a vulvar carcinoma following electron irradiation]. Geburtshilfe Frauenheilkd. 1985;45(7):494-6.
107. Kohler U, Schone M, Bilek K. Experience and results in the treatment of vulvar carcinoma relapses. [German]
Erfahrungen und Ergebnisse bei der Behandlung von Rezidiven des Vulvakarzinoms. Geburtshilfe und Frauenheilkunde. 1997;57(8):453-7.
108. Strauss HG, Lampe D. [Recurrent vulvar carcinoma at the Halle/S. University Gynecologic Clinic 1971-1990--diagnosis and results of therapy]. Zentralbl Gynakol. 1994;116(12):675-8.
109. Buchler DA, Kline JC, Tunca JC, Carr WF. Treatment of recurrent carcinoma of the vulva. GynecolOncol. 1979;8(2):180-4.
110. Miller B, Morris M, Levenback C, Burke TW, Gershenson DM. Pelvic exenteration for primary and recurrent vulvar cancer. GynecolOncol. 1995;58(2):202-5.
111. Kaur M, Joniau S, D'Hoore A, Van CB, Van LE, Leunen K, et al. Pelvic exenterations for gynecological malignancies: a study of 36 cases. IntJGynecolCancer. 2012;22(5):889-96.
112. Pathiraja P, Sandhu H, Instone M, Haldar K, Kehoe S. Should pelvic exenteration for symptomatic relief in gynaecology malignancies be offered? ArchGynecolObstet. 2014;289(3):657-62.
113. Tan KK, Pal S, Lee PJ, Rodwell L, Solomon MJ. Pelvic exenteration for recurrent squamous cell carcinoma of the pelvic organs arising from the cloaca--a single institution's experience over 16 years. Colorectal Dis. 2013;15(10):1227-31.
114. Blake P. Radiotherapy and chemoradiotherapy for carcinoma of the vulva. BestPractResClinObstetGynaecol. 2003;17(4):649-61.
115. Beriwal S, Shukla G, Shinde A, Heron DE, Kelley JL, Edwards RP, et al. Preoperative intensity modulated radiation therapy and chemotherapy for locally advanced vulvar carcinoma: analysis of pattern of relapse. IntJRadiatOncolBiolPhys. 2013;85(5):1269-74.
116. Moore DH, Ali S, Koh WJ, Michael H, Barnes MN, McCourt CK, et al. A phase II trial of radiation therapy and weekly cisplatin chemotherapy for the treatment of locally-advanced squamous cell carcinoma of the vulva: a gynecologic oncology group study. GynecolOncol. 2012;124(3):529-33.
117. Cormio G, Loizzi V, Gissi F, Serrati G, Panzarino M, Carriero C, et al. Cisplatin and vinorelbine chemotherapy in recurrent vulvar carcinoma. Oncology. 2009;77(5):281-4.
118. Tewari K, Cappuccini F, Syed AM, Puthawala A, DiSaia PJ, Berman ML, et al. Interstitial brachytherapy in the treatment of advanced and recurrent vulvar cancer. Am J Obstet Gynecol. 1999;181(1):91-8.
119. Perrone AM, Galuppi A, Cima S, Pozzati F, Arcelli A, Cortesi A, et al. Electrochemotherapy can be used as palliative treatment in patients with repeated loco-regional recurrence of squamous vulvar cancer: a preliminary study. Gynecol Oncol. 2013;130(3):550-3.

120. Gill BS, Bernard ME, Lin JF, Balasubramani GK, Rajagopalan MS, Sukumvanich P, et al. Impact of adjuvant chemotherapy with radiation for node-positive vulvar cancer: A National Cancer Data Base (NCDB) analysis. GynecolOncol. 2015;137(3):365-72.
121. Reade CJ, Eiriksson LR, Mackay H. Systemic therapy in squamous cell carcinoma of the vulva: current status and future directions. GynecolOncol. 2014;132(3):780-9.
122. Montana GS, Thomas GM, Moore DH, Saxer A, Mangan CE, Lentz SS, et al. Preoperative chemoradiation for carcinoma of the vulva with N2/N3 nodes: a gynecologic oncology group study. IntJRadiatOncolBiolPhys. 2000;48(4):1007-13.
123. Rose PG. Skin bridge recurrences in vulvar cancer: frequency and management. Int J Gynecol Cancer. 1999;9(6):508-11.
124. Durrant KR, Mangioni C, Lacave AJ, George M, van der Burg ME, Guthrie D, et al. Bleomycin, methotrexate, and CCNU in advanced inoperable squamous cell carcinoma of the vulva: a phase II study of the EORTC Gynaecological Cancer Cooperative Group (GCCG). GynecolOncol. 1990;37(3):359-62.
125. Horowitz NS, Olawaiye AB, Borger DR, Growdon WB, Krasner CN, Matulonis UA, et al. Phase II trial of erlotinib in women with squamous cell carcinoma of the vulva. Gynecol Oncol. 2012;127(1):141-6.



CHAPTER 5

Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: a review of the current literature

Marjolijn D. Trietsch*
Linda S. Nooij*
Katja N. Gaarenstroom
Mariette I.E. van Poelgeest

* Both authors contributed equally

Gynecologic Oncology 2015;136:143-157

Abstract

Vulvar cancer is a relatively rare gynecologic malignancy with an annual incidence in developed countries of approximately 2 per 100,000 women. Vulvar squamous cell carcinoma (VSCC) has two etiological pathways: a high risk human papillomavirus (HPV)-dependent route, which has usual vulvar intraepithelial neoplasia (uVIN) as a precursor lesion, and an HPV-independent route, which is associated with differentiated VIN (dVIN), lichen sclerosus, and genetic alterations, such as *TP53* mutations. Research on the molecular etiology of vulvar cancer has increased in past years, not only regarding genetic alterations, but also epigenetic changes. In genetic alterations, a mutation irreversibly changes the nucleotide sequence of the DNA, or the number of copies of chromosomes per cell is altered. In epigenetics, the nucleotide sequence remains the same but genes can be 'switched' on or off by, for example, DNA methylation or histone modification. We searched the current literature on genetic and epigenetic alterations in VSCC and its precursor lesions. Many studies have reported a higher incidence of somatic mutations in HPV-negative tumors compared to HPV-positive tumors, with *TP53* mutations being the most frequent. These somatic mutations seem to occur more often with increasing grades of dysplasia. Allelic imbalances or loss of heterozygosity are more frequently found in higher stages of dysplasia and in invasive carcinomas, but it is not exclusive to HPV-negative tumors. A limited number of studies are available on epigenetic changes in vulvar lesions, with hypermethylation of *CDKN2A* being the most frequently investigated change. For most genes, hypermethylation occurs more frequently in VSCC than in precursor lesions. As most studies have focused on HPV infection and *TP53* mutations, we suggest that more research should be performed using whole genome or next generation sequencing to determine the true landscape of genetic and epigenetic alterations in VSCC.

Introduction

Vulvar cancer is a rare malignant disease accounting for less than 5% of gynecological malignancies (1-3). The majority of these tumors are vulvar squamous cell carcinoma (VSCC). The annual incidence of VSCC in developed countries is two to three per 100,000 women and increases with age, with a peak incidence between 60 and 70 years of age (1, 4, 5).

The pathogenesis of VSCC can be subdivided into two different pathways: human papillomavirus (HPV)-dependent and HPV-independent (1-7). The HPV-dependent pathway accounts for 20-40% of VSCCs and has usual vulvar intraepithelial neoplasia (uVIN) as a precursor lesion (3, 4, 8). This pathway is more common in younger women and is associated with smoking, a higher number of sexual partners, and a compromised immune status (1, 3, 9). The incidence of VIN, especially the usual type, has increased in the last couple of years, even doubling in some countries (1, 4-6). The risk of the progression of a uVIN lesion towards VSCC seems low, occurring in 9-16% of patients who do not receive treatment and in approximately 3% of patients who have been treated (1, 6). However, some studies have reported a higher risk of progression (10, 11). The non-HPV pathway is associated with mutations in *TP53* and mainly occurs in older women (1-3, 6, 7). This pathway is associated with lichen sclerosus (LS), a chronic dermatosis associated with autoimmune diseases. Approximately 3-5% of women with LS progress towards VSCC (9, 12). Differentiated VIN (dVIN) is considered to be a precursor lesion of HPV-independent VSCC, with a higher malignant potential than uVIN (1, 6). dVIN can be difficult to diagnose for both clinicians and pathologists because of its subtle clinical and histological appearance (13). HPV-independent VSCC is associated with a worse prognosis than HPV-associated VSCC (3, 9). However, its carcinogenesis has not been fully clarified.

When diagnosed at an early stage, VSCC has a good prognosis, especially for patients without inguinofemoral lymph node metastasis at first presentation (14). Unfortunately, approximately one-third (15) of patients suffer from recurrent disease. In the latter group of patients, therapeutic options are limited due to severe morbidity associated with repeated treatment of local recurrences. Recurrent disease in inguinal lymph nodes has a very poor prognosis and is almost always fatal (16, 17). Information on genetic and epigenetic changes that play a role in the carcinogenesis of vulvar cancer may provide valuable insight into its etiology. Studies of many different types of cancer have shown that genetic and epigenetic alteration status can help predict prognosis and guide targeted therapy (18-23). For example, vemurafenib, a BRAF inhibitor, has shown clinical efficacy as targeted therapy for melanomas that harbor mutations in *BRAF* (24). In HPV-negative VSCC, mutations are often found in

the tumor suppressor gene *TP53* (1, 8, 9, 25, 26). *TP53* mutations are thought to be an early event in the development of VSCC because they are also found in dVIN and LS lesions (1, 6-8, 26). Other mutations have been described in VSCC and its precursor lesions, including mutations in the tumor suppressor genes *PTEN* and *CDKN2A* (27, 28). Other types of genetic alterations are allelic imbalances or copy number alterations, in which the number of copies of chromosomes per cell is altered. In addition to genetic mutations, epigenetic changes may also play a role in the development of VSCC. Epigenetic changes are defined as heritable changes in gene expression without changes in the DNA sequence. The best known epigenetic change is hypermethylation of CpG islands in the promoter regions of tumor suppressor genes, causing inactivation of the gene (19, 23, 29-32). In vulvar cancer, hypermethylation of the promoters of *RASSF2A*, *MGMT,* and *TSP1* has been described (30). Here, we review the current literature and summarize the current understanding of the role of genetic and epigenetic changes in VSCC and its precursor lesions.

Materials and methods

Relevant studies on genetic alterations (somatic mutations, allelic imbalances, loss of heterozygosity, copy number changes, and microsatellite instability) and epigenetic changes (hypomethylation and hypermethylation, microsatellite instability, and chromatin, histone, and posttranscriptional modifications) were identified from an extensive search on PubMed, Embase, Web of Science, Cochrane, and ScienceDirect. After consulting a medical librarian, a combination of Medical Subject Headings (MeSH) and free text words were formulated. Our search included the terms vulvar neoplasm, vulvar carcinoma, vulvar intraepithelial neoplasia, lichen sclerosus et atrophicus, mutation, microsatellite instability, genetic, epigenetic, hypermethylation, chromatin, histone, and posttranscriptional modifications. Research published until 31 July 2014 that studied somatic mutations and epigenetic changes in VSCC, VIN, and/or LS were included in this review. Exclusion criteria were languages other than English, Dutch, German, French, or Italian, meeting abstracts, or if the researchers only performed immunohistochemistry to evaluate protein function. Two researchers (MDT and LN) independently assessed all articles based on the title, abstract, or full article. Articles for which there was disagreement regarding inclusion or exclusion were discussed and a consensus reached. The electronic search was complemented by a manual search of bibliographies from relevant articles in order to identify additional relevant studies not encountered in the electronic search. The articles that met all inclusion criteria are described in this review.

Results

The electronic search identified 198 articles on genetic alterations in VSCC, VIN, and LS. The manual search yielded another 17 articles. 59 of these articles met the inclusion criteria and were included in this review (Tables 1 and 3). For epigenetic changes in VSCC, VIN, and LS, we found 49 articles, nine of which are included in this review (Table 4). Four articles reported on both genetic and epigenetic changes and are found in both table 1 and table 4 (28, 33-35). A flowchart illustrating the inclusion and exclusion of articles is shown in figure 1.

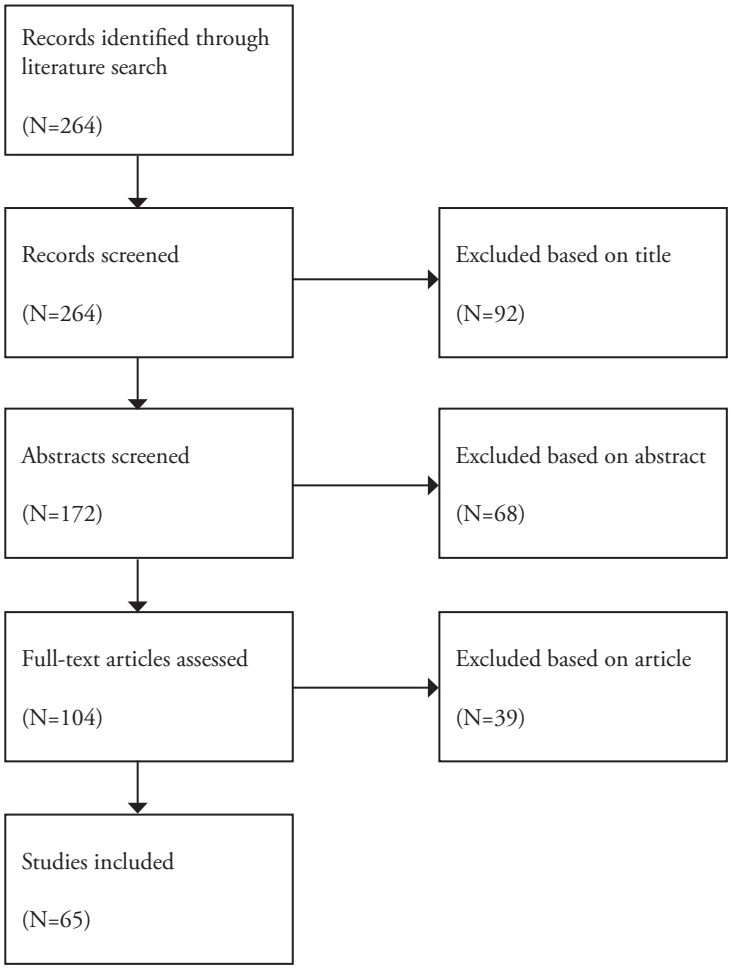


Figure 1: Inclusion and exclusion of articles

Table 1: Studies on mutations in vulvar cancer and its precursors

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
Pilotti	1993	5	verrucous VC	-	*TP53*	0%	SSCP exon 5-9 + confirmation sequencing	
Kurvinen	1994	1	CIS	+	*TP53*	0%	SSCP exon 5-9 + confirmation sequencing	
		1	VIN	+	*TP53*	0%		
		2	VSCC	-	*TP53*	0%		
		7	VSCC	+	*TP53*	0%		
Lee	1994	9	VSCC	-	*TP53*	44%	SSCP exon 5-8 and part of exon 4	
		12	VSCC	+	*TP53*	8%		
Milde-Langosch	1995	12	VIN	50%*	*TP53*	33%	PCR-TGGE	* not described in association to mutations
Pilotti	1995	7	VIN*	+	*TP53*	0%	SSCP exon 5-9	*some adjacent to reported VSCC
		12	VSCC	-	*TP53*	33%		
		4	VSCC	+	*TP53*	50%		
Kim	1996	11	VSCC	-	*TP53*	36% (25% keratinising, 100% Pagets)	SSCP exon 5-8	* 11 (8 keratinising, 1 basaloid, 2 Pagets) 7 (3 keratinising, 2 basaloid, 1 Pagets, 1 warty)
		7	VSCC	+	*TP53*	0%		
Sliutz	1997	38	VSCC	not tested	*TP53*	32%	PCR-TGGE	
Wong	1997	6	VSCC	not tested	*CDKN2A* and *CDKN2B*	0%	SSCP *CDKN2A* exon 1-3 and *CDKN2B* exon 1-2	
Flowers	1999	10*	VIN	-	*TP53*	10%		* multiple samples from same patient

		11*	VIN	+	*TP53*	9%		
		15	VSCC	-	*TP53*	29% KSC, 0% basaloid		
		15	VSCC	+	*TP53*	33% KSC, 8% basaloid		
Ngan	1999	25	VSCC	-	*TP53*	20%	SSCP exon 5-8 + confirmation sequencing	
		23	VSCC	+	*TP53*	22%		
Brooks	2000	23	VSCC	-	*TP53*	74%	SSCP exon 4-9	codon 72P/R same cohort as Marin 2000 and O'Nion 2001
		13	VSCC	+	*TP53*	31%		
Holway	2000	2*	VIN	not tested	*PTEN*	100%	SSCP exon 5-8	* same patients as VSCC
		10	VSCC	not tested	*PTEN*	60%		1 patient had PTEN mutation in VIN but not in adjacent VSCC. In 3 patients different mutations were found in VIN and VSCC
Marin	2000	36	VSCC	not tested	*TP53*	58%	SSCP exon 4-9 + confirmation sequencing	
		10	LS	-	*TP53*	70%		
		29 (3 basaloid, 26 squamous)	VC	-	*TP53*	55%		
		11 (3 basaloid, 8 squamous)	VC	+	*TP53*	45%		
Wada	2000	1	VIN	+	*TP53* + *KRAS*	0% *TP53*, 0% *KRAS*	SSCP *TP53* exon 5-8, KRAS exon 1	
O'Nions	2001	23	VSCC	-	*TP53* + *CDKN2A*	74% *TP53*, 13% *CDKN2A*	SSCP CDKN2A exon 1α + 2, TP53 exon 7-9	
		13	VSCC	+	*TP53* + *CDKN2A*	31% *TP53*, 0% *CDKN2A*		

Gasco	2002	23	VSCC	-	*CDKN2A* + *Stratifin* +*TP53*	13% *CDKN2A*, 0% *Stratifin*, 73,9 % *TP53*		
		20	VIN	-	*CDKN2A* + *Stratifin* +*TP53*	0% *CDKN2A*, 0% *Stratifin*, 0% *TP53*		*CDKN2A* and *stratifin* were tested on 11 patients
		12	VIN	+	*CDKN2A* + *Stratifin* + *TP53*	0% *CDKN2A*, 0% *Stratifin*, 0% *TP53*		*CDKN2A* and *stratifin* were tested on 11 patients
		13	VSCC	+	*CDKN2A* + *Stratifin* +*TP53*	0% *CDKN2A*, 0% *Stratifin*, 30,8% *TP53*		
Rampone	2002	8	LS	not tested	*TP53*	63%	Sanger sequencing exon 5-9	
		10	LSC	not tested	*TP53*	0%		
Reddy	2002	32	VIN	not tested	*CHK2*	0% *CHK2*		
		40	VSCC	not tested	*CHK2* + *TP53*	5 % *CHK2*, 100% *TP53* *	SSCP *CHK2* exon 1a, 1b, 2-14, TP53	* only tested in *CHK2* mutated samples
Vanin	2002	62*	LS	-	*TP53*	5%	Sanger sequencing exon 5-8	* 25 with VSCC, 37 without VSCC
		29	VSCC	-	*TP53*	28%		
Rolfe	2003	12	LS	not tested	*TP53*	58%	Sanger sequencing exon 5-8	
		27	VSCC	not tested	*TP53*	81%		
Almeida	2004	2	undifferentiated VIN	-	*TP53*	50%	SCCP exon 5-8	
		6	undifferentiated VIN	+	*TP53*	17%		
Chulvis do Val	2004	13	undifferentiated VIN	64%*	*TP53*	38%	SSCP exon 5-8	* not described in association to mutations
Olawaiye	2007	2	VSCC	not tested	*EGFR*	0%	Sanger sequencing exon 18-24	
Osakabe	2007	16	VSCC	-	*TP53*	63%	SCCP exon 5-8	

		5	VSCC	+	*TP53*	20%		
		7	Bowenoid early invasion and 1 invasive SCC	+	*TP53*	0%		
Soufir	2007	21	LS	not tested (not for all)	*CDKN2A* + *TP53*	0% *CDKN2A*, 0% *TP53*	SSCP *CDKN2A* exon 1α, 1β and 2, *TP53* exon 4-9	
		2	VIN	not tested (not for all)	*CDKN2A* + *TP53*	0% *CDKN2A*, 0% *TP53*		
		5	VSCC	not tested (not for all)	*CDKN2A* + *TP53*	20% *CDKN2A*, 60% *TP53*		
Tapp	2007	224	LS	not tested	*TP53* + *KRAS* (2+1 hotspot codons only)	0% had a single mutant population that exceeded 20 per 10^6	PCR/RE/LCR	reports SBS single base instability (not somatic mutations, but 1 in a million errors) and only looked at 2 hotspots in *TP53* (codon 248 and 273) and 1 in *KRAS* (codon 12)
Aulman	2008	12	VIN (7 uVIN, 5 dVIN)	-	*TP53*	17%	SSCP exon 4-10	
		20	uVIN	+	*TP53*	0%		
		24	VSCC	-	*TP53*	17%		
		4	VSCC	+	*TP53*	0%		
Growdon	2008	19	VSCC	-	*EGFR*	0%	Sanger sequencing exon 18-21	
		22	VSCC	+	*EGFR*	0%		
		5*	CIS	not tested	*PTEN*	60%		
Pinto	2010	11	VIN	-	*TP53*	60%	Sanger sequencing	
		5	VSCC	-	*TP53*	80%		

Choschzick	2011	21	VSCC	-	*TP53*	77%	Sanger sequencing exon 5-8	
		18	VSCC	+	*TP53*	24%		
Janku	2011	2	VSCC	not tested	*PIK3CA*	0%	Sanger sequencing c532-554 of exon 9 and c1011-1062 of exon 20	
Horowitz	2012	17	VSCC	not tested	*EGFR*	0%	Sanger sequencing	
Gambichler	2013	10	LS	not tested	*TP53, NRAS, KRAS, IDH1, IDH2, TET2*	0%	Sanger sequencing *IDH1* exon 4, *IDH2* exon 4, *TET2* exon 3 + 11, *TP53* exon 4,6,7, *KRAS* codon 12, *HRAS* exon 3, *NRAS* exon 2-3	
		5	CIS	-	*EGFR*	0%		
		5	CIS	+	*EGFR*	0%		
Trietsch	2014	89	VSCC*	-	*BRAF*, *CDKN2A*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN*, and *TP53*	0% *BRAF*, 16% *CDKN2A*, 0% *CTNNB1*, 0% *FBXW7*, 0% *FGFR2*, 0% *FGFR3*, 0% *FOXL2*, 11% *HRAS*, 1% *KRAS*, 0% *NRAS*, 8% *PIK3CA*, 3% *PPP2R1A*, 1% *PTEN*, 62% *TP53*	Hot spot mass spectrometry, Sanger sequencing *TP53 exon 5-9*	*Partial overlap in VSCC patients reported in a recent article by Spaans et al. (1)

		18	VSCC*	+	*BRAF*, *CDKN2A*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN*, and *TP53*	0% *BRAF*, 0% *CDKN2A*, 0% *CTNNB1*, 0% *FBXW7*, 0% *FGFR2*, 0% *FGFR3*, 0% *FOXL2*, 0% *HRAS*, 0% *KRAS*, 0% *NRAS*, 0% *PIK3CA*, 0% *PPP2R1A*, 0% *PTEN*, 17% *TP53*

HPV: human papillomavirus
N: number
LS: lichen sclerosus
LSC: lichen sclerosus chronicans
VSCC: vulvar squamous cell carcinoma
VIN: vulvar intraepithelial neoplasia
uVIN: usual vulvar intraepithelial neoplasia
dVIN: differentiated vulvar intraepithelial neoplasia
CIS: carcinoma in situ
SCCP: single strand confirmation polymorphism
PCR: polymerase chain reaction
TGGE: temperature gradient gel electrophoresis
KSC: keratinizing squamous carcinoma
LCR: ligand chain reaction
RE: restriction endonuclease
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for mutations

Table 2: Overall mutation frequencies

	LS				VIN				VSCC							
	HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown	
TP53	10/72	14%	12/285	4%	2/66	3%	10/47	21%	11/29	38%	28/171	16%	109/361	30%	28/108	26%
PTEN									2/2	100%	0/18	0%	1/89	1%	6/10	60%
EGFR											0/22	0%	0/19	0%	0/19	0%
BRAF											0/18	0%	0/89	0%		
HRAS											0/18	0%	10/89	11%		
KRAS			0/10	0%							0/18	0%	1/89	1%		
NRAS			0/10	0%							0/18	0%	0/89	0%		
CDKN2A			0/21	0%	0/4	0%	0/2	0%	0/2	0%	0/44	0%	20/135	15%	1/11	9%
CTNNB1											0/18	0%	0/89	0%		
PPP2R1A											0/18	0%	3/89	3%		
FBXW7											0/18	0%	0/89	0%		
PIK3CA											0/18	0%	7/89	8%	0/2	0%
IDH1			0/10	0%												
IDH2			0/10	0%												
TET2			0/10	0%												
CHK2									0/32	0%					2/40	5%
FGFR2											0/18	0%	0/89	0%		
FGFR3											0/18	0%	0/89	0%		
FOXL2											0/18	0%	0/89	0%		
Stratifin					0/4	0%	0/2	0%			0/13	0%	0/23	0%		

LS: lichen sclerosus
VIN: vulvar intraepithelial hyperplasia
VSCC: vulvar squamous cell carcinoma
HPV: human papillomavirus
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for mutations

Somatic mutations

A total of 34 articles were included that described somatic mutations (Table 1) (8, 25-28, 33-61). Mutations were most often studied and detected in *TP53*, with frequencies of up to 70% for LS, 60% for VIN, and 81% for vulvar cancer. *CDKN2A* mutations were not detected in LS or VIN, but occurred in 0-60% of VSCCs. Table 2 shows the overall frequencies of mutations for all included studies. HPV-negative tumors harbored more mutations than HPV-positive tumors, and the percentage of mutated samples gradually increased with higher stages of (pre)cancerous lesions.

Allelic imbalances, loss of heterozygosity, and copy number changes

A total of 24 articles were included that reported allelic imbalances or copy number changes in vulvar cancer and its precursors (Table 3) (36, 45, 47-49, 51, 52, 55, 56, 58, 60, 62-73). Allelic imbalances occurred most often on chromosomes 3, 8, 11, 13, and 17. Three studies focused on the total DNA index, and each found high percentages of aneuploidy and tetraploidy (62-64). Bryndorf was the only one to test HPV infection and found the highest percentage of aneuploidy and tetraploidy in HPV-negative VSCC. Allelic imbalances were more frequently observed in higher stages of both precancerous and cancerous lesions (63).

Microsatellite instability

We included three articles that reported on microsatellite instability (MSI) (65, 74, 75), a condition in which repetitive DNA sequences are susceptible to errors because the Mismatch Repair system is not functioning properly (Table 4). The articles by Bujko and Lin looked at MSI in HPV-positive and negative VSCC. Bujko et al. found no MSI in the 44 patients they investigated (29 HPV-negative and 15 HPV-positive) (74). Lin reported MSI in locus 3.1 in one of two patients with HPV-positive VSCC (65). Pinto et al. focused on MSI and allelic imbalances in uVIN, dVIN and LS, and found that MSI was confined exclusively to HPV-negative dVIN and LS lesions, but did not occur in the 15 uVINs they studied (75). The data by Pinto suggest that these molecular changes are possibly early events in the HPV-independent route of vulvar carcinogenesis, and that MSI may play a role in the malignant potential of LS. However, in a small cohort of 4 patients with VSCC described by Lin et al., 2 patients with HPV-positive tumors displayed MSI as well. These data indicate that the exact role of MSI in vulvar carcinogenesis needs to be elucidated.

Table 3: Studies on allelic imbalances in vulvar cancer and its precursors

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
Wong	1997	6	VSCC	not tested	*CDKN2A* and *CDKN2B*	50% *CDKN2A*, 50% *CDKN2B*	loss	LOH	
Lin	1998	2	VIN	-		0% 1.2, 0% 2.3, 50% 2.4, 0% 3.1, 0% 3.4, 0% 4.1, 50% 5.2, 50% 5.3, 0% 8.2, 0% 21.1	loss	LOH	
		2	VIN	+		0% 1.2, 50% 2.3, 50% 2.4, 0%3.1, 50% 3.4, 0% 4.1, 0% 5.2, 0% 5.3, 50% 8.2, 0% 21.1	loss		
		2	VSCC	-		0% 1.2, 100% 2.3, 100% 2.4, 50% 3.1, 50% 3.4, 50% 4.1, 100% 5.2, 50% 5.3, 50% 8.2, 50% 21.1	loss		

		2	VSCC	+		50% 1.2, 0% 2.3, 100% 2.4, 0% 3.1, 0% 3.4, 0% 4.1, 0% 5.2, 100% 5.3, 50% 8.2, 0% 21.1	loss		
Flowers	1999	10*	VIN	-	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	54% 3p, 14% 13q (*RB*), 9% 17p (*TP53*)	loss	LOH	* multiple samples from same patients
		10*	VIN	+	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	16% 3p, 6% 13q (*RB*), 0% 17p (*TP53*)	loss		
		15	VSCC	-	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	93% 3p, 27% 13q (*RB*), 62% 17p (*TP53*)	loss		
		15	VSCC	+	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	67% 3p, 31% 13q (*RB*), 15% 17p (*TP53*)	loss		

Scheistroen	1999	167	VSCC	not tested	77 % diploid, 23% aneuploid		FACS
Pinto	1999	8	VSCC	-	Overall 36% LOH. Most frequent: 83% 5q, 100% 10p, 29% 1p, 25% 2q, 50% 3p, 63% 8p, 63% 8q, 60% 10q, 50% 11q, 29% 15q, 80% 17p, 50% 21q, 60% 22q.	loss	LOH
		8	VSCC	+	Overall 30% LOH. Most frequent: 13% 5q, 17% 10q, 33% 1p, 0% 2q, 50% 3p, 13% 5q, 33% 8p, 50% 8q, 17% 10p, 25% 11q, 43% 15q, 43% 17p, 67% 21q, 20% 22q	loss	

Pinto	2000	16	VIN (5 uVIN, 11 dVIN)	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	15%*	both	LOH	*scoring informative (heterozygous) loci
		14	VIN (10 uVIN, 4 dVIN)	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	25%*	both		*scoring informative (heterozygous) loci
		17	LS	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	10%*	both		*scoring informative (heterozygous) loci
Brooks	2000	23	VSCC	-	*TP53*	61%	loss	LOH	codon 72P/R same cohort as Marin 2000 and O'Nion 2001
		13	VSCC	+	*TP53*	54%	loss		
Carlson	2000	12	LS	not tested	chr 17	chr 17 aneusomy:100%. DNA index aneuploidy: 58%		FISH	
		3	VIN	not tested	chr 17	chr 17 aneusomy: 100% DNA index aneuploidy: 67%			
		14*	VSCC	not tested	chr 17	chr 17 aneusomy: 93%. DNA index aneuploidy: 86%			* 10 SCC, 4 SCCIS
Marin	2000	36	VSCC	not tested	*TP53*	54%	loss	LOH	
Wada	2000	1	VIN	+	3p14.2, 3p, 9p21, 9p23, 13q22, 17p12	0%	loss	LOH	

Jee	2001	10	VSCC	not tested	DNA copy number changes in 80%. Loss: 50% 4p13-pter, 40% 3p, 10% 5q14-q23, 10% 6q11-q16, 10% 11q21-qter, 10% 13q14-q32. Gain: 40% 3q, 30% 8q, 10% 9p, 10% 14, 10% 17, 10% 20q	both	CGH
Rosenthal	2001	13	VSCC	-	LOH of 48% 17p, 40% 9p, 48% 3p, 44% 4q, 43% 5p, 44% 11p	loss	
		54	VSCC	+	LOH of 48% 17p, 40% 9p, 48% 3p, 44% 4q, 43% 5p, 44% 11p	loss	
Allen	2002	8	VSCC	-	Most common: 75% 8q gain, 0% 3q gain, 13% 3p loss, 50% 11q loss	both	CGH

		10	VSCC	+		Most common 20% 8q gain, 50% 3q gain, 40% 3p loss, 40% 11q loss	both		
Reddy	2002	32	VIN	not tested	*CHK2*	0%*			
		40	VSCC	not tested	*CHK2*	2%*	loss	direct sequencing of RT-PCR product	* only tested in *CHK2* mutated samples
Vanin	2002	62*	LS	-	*TP53*	0%	loss	LOH	* 25 with VSCC, 37 without VSCC
		29	VSCC	-	*TP53*	74%	loss		
Bryndorf	2004	4	condyloma	-		0 chromosomal abberations	both	hrCGH and FACS	
		2	VIN	-		100% diploid. Most common gain of: 0% chr 1, 0% 3q, 0% 20q, 0% 20p, 0% 3q, 0% 8q. Loss of 0% 3p, 0% 8p	both		

	9	VIN	+	40% diploid, 30% aneuploid, 30% tetraploid. Most common gain of: 60% chr 1, 50% 3q, 50% 20q, 40% 20p, 30% 8q. Loss of 20% 3p, 0% 8p	both
	6	VSCC	-	25% diploid, 75% aneuploid. Most common gain of: 0% chr 1, 75% 3q, 50% 20q, 50% 20p, 100% 8q. Loss of 50% 3p, 50% 8p	both
	4	VSCC	+	50% diploid, 50% tetraploid. Most common gain of: 0% chr 1, 66% 3q, 17% 20q, 17% 20p, 33% 8q. Loss of 83% 3p, 33% 8p	both

Huang	2005	8	VSCC	75%*		gains of 1q 13%, 3q 38%, 5p 38%, 8q 75%. Losses 3p 38%, 4p 13%, 11p 13%	both	CGH	* not described in association to genetic changes
Olawaiye	2007	2	VSCC	not tested	*EGFR*	0%		q rtPCR	
Osakabe	2007	16	VSCC	-		LOH of 44% 3p14.2 (*FHIT*), 38% 3p26 (*VHL*), 38% 5q31 (*APC*), 63% 9q21 (*p16*), 67% 9q22.3 (*PTECH*), 38% 10p15 (*PAHX*), 30% 13q14.3-21.1 (*Rb*), 40% 17p13 (*TP53*), 44% 18q21 (*DCC*). Fractional allelic loss 43%	loss	LOH	
		5	VSCC	+		LOH of 50% 3p14.2 (*FHIT*), 100% 9q21 (*p16*), 50% 9q22.3 (*PTCH*), Fractional allelic loss 18%	loss		
Yangling	2007	10	VSCC	-	3q, 3p, 4p, 8q, 12q	Gain: 10% 3q, 70% 8q 0% 12q, Loss: 40% 3p, 50% 4p	both	CGH	

		11	VSCC	+	3q, 3p, 4p, 8q, 12q	Gain: 73% 3q, 64% 12q, 9% 8q. Loss: 46% 3p, 55% 4p	both		
Growdon	2008	19	VSCC	-	*EGFR* + *HER2*	32% *EGFR*, 0% *HER2*, 16% polysomy chr 7	gene amplification	FISH	
		22	VSCC	+	*EGFR* + *HER2*	0% *EGFR*, 0% *HER2*			
		5	CIS	-	*EGFR* + *HER2*	0% *EGFR*, 0% *HER2*			
		5	CIS	+	*EGFR* + *HER2*	0% *EGFR*, 0% *HER2*			
Aulman	2008	12	VIN (7 uVIN, 5 dVIN)	-	3q26	73%	gain	FISH	
		20	uVIN	+	3q26	50%	gain		
		24	VSCC	-	3q26	83%	gain		
		4	VSCC	+	3q26	75%	gain		
Horowitz	2012	17	VSCC	not tested	*EGFR*	12%	gene amplification	FISH	
Lavorato-Rocha	2013	139	VSCC	33%*	*TP53*	65% normal gene / chr copy number, 19% polysomy, 9% monosomy, 6% deletion	both	FISH	* not described in association to genetic changes

Micci	2013	14	VSCC	not tested	Amongst others *FHIT*, *PTPRD*	70% aneuploid, 20% tetraploid, 10% diploid. 90% array-CGH imbalances. Loss of a region of 64% 8p23.1, 57% 8p21.3, 57% 8p12, 50% 3p14.2, 50% 3p13, 50% 8p23.3-p23.1, 50% 8p23.1-p11.23, 50% 8p11.22-p11.1, 50% 8q23.3, 50% 8q24.12-q24.22, 50% 9p23. Homozygous deletion of 29% p23 (*PTPRD*). No common amplified region.	both	arrayCGH + rtPCR + karyotyping

HPV: human papillomavirus
N: number
LS: lichen sclerosus
LSC: lichen sclerosus chronicans
SCC: vulvar squamous cell carcinoma
VIN: vulvar intraepithelial neoplasia
AI: allelic imbalance
LOH: loss of heterozygosity
FISH: fluorescence in situ hybridization
RT-PCR: real time polymerase chain reaction
(hr)CGH: (high resolution) comparative genomic hybridization
FACS: fluorescence-activated cell sorting
SCCIS: squamous cell carcinoma in situ
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for allelic imbalances

Table 4: Studies on microsatellite instability (MSI) in vulvar cancer and its precursors

Author	Year	No. of patients	Diag-nosis	HPV-status	Locus	% MSI	Technique used
Lin	1998	2	VSCC	-	3.1	0%	PCR
		2	VSCC	+	3.1	50%	
Bujko	2012	29	VSCC	-		0%	PCR
		15	VSCC	+		0%	
Pinto	2000	5	uVIN	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	PCR
		10	uVIN	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	
		11	dVIN	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	27%	
		4	dVIN	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	
		17	LS	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	12%	

HPV: human papillomavirus
LS: lichen sclerosus
VSCC: vulvar squamous cell carcinoma
VIN: vulvar intraepithelial neoplasia
PCR: polymerase chain reaction

Epigenetic alterations

Nine articles were included that reported on epigenetic alterations in VSCC or its precursors (Table 5) (28-30, 33, 34, 76-79). *CDKN2A* was studied most often (28-30, 33, 34, 76, 78, 79). *CDKN2A* is more frequently hypermethylated in VSCC (up to 68%) and VIN (up to 72%) than in LS (up to 47%), but there is great variability in the reported frequencies. An overview of all genes tested for hypermethylation and the percentage of hypermethylation is shown in table 6. When HPV status was not specified for all genes tested for hypermethylation, HPV status was interpreted as unknown.

Table 5: Studies on hypermethylation in vulvar cancer and its precursors

Author	Year	No. of patients	Diag-nosis	HPV-status	Gene	% Hypermethylation	Technique used	Remarks
O'Nions	2001	13	VSCC	HPV 16 +	*CDKN2A*	15,4%	msPCR	
		23	VSCC	HPV 16 -	*CDKN2A*	47,8%	msPCR	
Gasco	2002	0	VIN 1	HPV 16 +	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		4	VIN 1	HPV 16 -	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		1	VIN 2	HPV 16 +	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		5	VIN 2	HPV 16 -	*Stratifin, CDKN2A*	40% *Stratifin*, 40% *CDKN2A*	msPCR	
		11	VIN 3	HPV 16 +	*Stratifin, CDKN2A*	45,5% *Stratifin*, 9,1% *CDKN2A*	msPCR	
		11	VIN 3	HPV 16 -	*Stratifin, CDKN2A*	72,7% *Stratifin*, 72,7% *CDKN2A*	msPCR	
		13	VSCC	HPV 16 +	*Stratifin, CDKN2A*	53,8% *Stratifin*, 15,4% *CDKN2A*	msPCR	
		23	VSCC	HPV 16 -	*Stratifin, CDKN2A*	56,5% *Stratifin*, 47,8% *CDKN2A*	msPCR	
Lerma	2002	21	LS	not tested	*CDKN2A*	42,8%	ms-PCR	
		13	9 uVIN, 4 dVIN	not tested	*CDKN2A*	69,2%	ms-PCR	
		38	VSCC	not tested	*CDKN2A*	68%	ms-PCR	

Soufir	2007	2	LS	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		8	LS	HPV 16 -	*CDKN2A, p14*	12,5% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VIN3	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VSCC	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VSCC	HPV 16 -	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
Aide	2010	15	LS	not tested	*DAPK* + *CDKN2A*	13% *DAPK*, 47% *CDKN2A*	ms-PCR	
Guerrero	2011	21	LS not associated with VSCC	HPV + 25%	*RASSF1A, RASSF2A, CDKN2A, TSP-1* and *MGMT*	52,4% *RASSF1A*, 0% *RASSF2A*, 19% *CDKN2A*, 52,4% *TSP-1*, 0% *MGMT*	ms-PCR	25% HPV positive, but HPV status not specified per gene investigated for hypermethylation
		12	LS associated with VSCC	not tested	*RASSF1A, RASSF2A, CDKN2A, TSP-1* and *MGMT*	33,3% *RASSF1A*, 8,3% *RASSF2A*, 16,6% *CDKN2A*, 50% *TSP-1*, 41,7% *MGMT*	ms-PCR	
		1	VSCC	HPV +	*RASSF1A, RASSF2A, CDKN2A, TSP-1* and *MGMT*	0% *RASSF1A*, 0% *RASSF2A*, 0% *CDKN2A*, 20% *TSP-1*, 0% *MGMT*	ms-PCR	TSP-1 hypermethylation was tested on 5 patients
		11	VSCC	HPV -	*RASSF1A, RASSF2A, CDKN2A, TSP-1* and *MGMT*	45,5% *RASSF1A*, 72,7% *RASSF2A*, 54,5% *CDKN2A*, 40% *TSP-1*, 72,7% *MGMT*	ms-PCR	TSP-1 hypermethylation was tested on 25 patients

Aide	2012	23	LS	not tested	*DAPK* + *CDKN2A*	17% *DAPK*, 35% *CDKN2A*	ms-PCR	
Oonk	2012	20	VSCC	not tested	*CDKN2A, MGMT, TWIST1, CADM1, TERT* and *TFPI2*	65% *CDKN2A*, 45% *MGMT*, 35% *TWIST 1*, 55% *CADM1*, 100% *TERT*, 60% *TFPI2*	msPCR	
Guerrero	2013	21	LS	HPV + 25%	*TSLC-1*	25% *TSLC-1*	ms-PCR	25% HPV positive, but HPV status not specified per gene investigated for hypermethylation
		30	VSCC	16,7% +	*TSLC-1*	44,4% *TSLC-1*	ms-PCR	Same cohort as Guerrero 2011. Only new results are described here.

HPV: human papillomavirus
LS: lichen sclerosus
LSC: lichen sclerosus chronicans
VSCC: vulvar squamous cell carcinoma
VIN: vulvar intraepithelial neoplasia
msPCR: methylation-specific polymerase chain reaction
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for hypermethylation

Table 6: Overall hypermethylation frequencies

	LS						VIN						VSCC					
	HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown	
CDKN2A	0/2	0%	1/8	12,5%	26/92	28,3%	1/14	7,1%	10/20	50%	9/13	69,2%	4/29	13,8%	28/59	47,5%	39/58	67,2%
p14	0/2	0%	0/8	0%			0/2	0%					0/2	0%	0/2	0%		
DAPK					6/38	15,8%												
MGMT					0/33	0%											9/20	45%
TWIST1																	7/20	35%
CADM1																	11/20	55%
TERT																	20/20	100%
TFPI2																	12/20	60%
RASSF1A					15/33	45,5%												
RASSF2A					1/33	3,0%												
TSP-1					17/33	51,5%												
Stratifin							5/12	41,7%	10/20	50%			7/13	53,8%	11/23	56,5%		
TSLC-1					9/21	42,9%											11/30	44,4%

LS: lichen sclerosus
VIN: vulvar intraepithelial hyperplasia
VSCC: vulvar squamous cell carcinoma
HPV: human papillomavirus
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for hypermethylation

Discussion

A growing body of research has focused on genetic and epigenetic changes in vulvar cancer. The combined results of the currently available literature on genetic and epigenetic changes confirm the hypothesis that HPV and *TP53* mutations play almost separate, but key roles in the carcinogenesis of VSCC (Table 5). Patients infected with HPV are less likely to carry somatic mutations than patients without HPV, but allelic imbalances seem to occur in both groups. The cumulative number of genetic changes increases with increasing grade of dysplasia and cancer stage. Although only a few studies have sufficient numbers of patients to perform survival analysis related to genetic and epigenetic changes, the findings suggest that tumors harboring a mutation, which are most often HPV-independent VSCC, have a worse prognosis than VSCC without (epi) genetic changes (36, 43, 50, 54, 58, 62, 73, 80).

The frequencies of detected mutations vary between studies. These differences can be explained, in part, by the composition of the cohorts. The included cohorts may vary in terms of age and ethnic background or tumor stage, which is known to be related to genetic alterations. Also, differences in the techniques used and coverage of the screened exons may play a role. Detection methods have improved over the last few decades, which is reflected in an overall increase in the number of detected *TP53* mutations within HPV-negative tumor samples.

The amount of research on epigenetic changes in VSCC and its precursors is limited, but studies in other types of cancer have shown the importance of these tumor characteristics in the development of targeted therapy (81). We only found articles on hypermethylation. In our literature search we did not find any articles on other possible epigenetic changes in VSCC or its precursors, such as chromatin remodeling or histone modifications. Most research on hypermethylation has studied different genes so a comparison cannot be made. Only *CDKN2A* has been investigated by more than one group. The hypermethylation frequencies that were found differ greatly between LS, VIN, and VSCC. The trend appears to be more hypermethylation in VSCC, but with the limited data it is difficult to draw any conclusions. With the fast development of research techniques focusing on epigenetic alterations in tumors, and the knowledge already gained on targeted therapy for epigenetically altered tumors, future research on this topic is promising.

In conclusion, genetic and epigenetic changes are detected more often with increasing precursor and tumor stage, and are more frequently found in HPV-negative patients than HPV-positive patients. However, compared to other types of cancer, studies on genetic and epigenetic changes in vulvar cancer and its precursors is relatively few and,

therefore, our knowledge on this subject is still limited. Most genetic studies focus on HPV infection and TP53 mutations, , the latter being the most frequent genetic change found in human cancers so far. Recent studies provide evidence that somatic mutations often do occur in other genes, such as CDKN2A and HRAS. Of all premalignant and malignant vulvar lesions, HPV-independent VSCC represents the largest group of patients with the worst prognosis and most difficulties in the diagnosis and treatment of progressive tumors. The upcoming availability of screening methods for somatic mutations that provide information on the complete or very large parts of the genome, such as next generation sequencing, may provide us with more insight into the mutational and epigenetic landscape and the etiology of vulvar cancer. Hopefully, these advances will increase future treatment possibilities and improve prognosis.

Conflict of interest statement

There are no conflicts of interest.

References

1. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. Histopathology. 2013;62(1):161-75.
2. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
3. van der Avoort IA, Shirango H, Hoevenaars BM, Grefte JM, de Hullu JA, de Wilde PC, et al. Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways. IntJGynecolPathol. 2006;25(1):22-9.
4. Schuurman MS, van den Einden LC, Massuger LF, Kiemeney LA, van der Aa MA, de Hullu JA. Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma. EurJCancer. 2013;49(18):3872-80.
5. van de Nieuwenhof HP, Massuger LF, van der Avoort IA, Bekkers RL, Casparie M, Abma W, et al. Vulvar squamous cell carcinoma development after diagnosis of VIN increases with age. EurJCancer. 2009;45(5):851-6.
6. McCluggage WG. Premalignant lesions of the lower female genital tract: cervix, vagina and vulva. Pathology. 2013;45(3):214-28.
7. Raspollini MR, Asirelli G, Moncini D, Taddei GL. A comparative analysis of lichen sclerosus of the vulva and lichen sclerosus that evolves to vulvar squamous cell carcinoma. AmJObstetGynecol. 2007;197(6):592-5.
8. Pinto AP, Miron A, Yassin Y, Monte N, Woo TY, Mehra KK, et al. Differentiated vulvar intraepithelial neoplasia contains Tp53 mutations and is genetically linked to vulvar squamous cell carcinoma. ModPathol. 2010;23(3):404-12.
9. Hantschmann P, Sterzer S, Jeschke U, Friese K. P53 expression in vulvar carcinoma, vulvar intraepithelial neoplasia, squamous cell hyperplasia and lichen sclerosus. Anticancer Res. 2005;25(3A):1739-45.
10. Jones RW, Rowan DM, Stewart AW. Vulvar intraepithelial neoplasia: aspects of the natural history and outcome in 405 women. Obstet Gynecol. 2005;106(6):1319-26.
11. Jones RW, Rowan DM. Vulvar intraepithelial neoplasia III: a clinical study of the outcome in 113 cases with relation to the later development of invasive vulvar carcinoma. ObstetGynecol. 1994;84(5):741-5.
12. Rolfe KJ, Eva LJ, MacLean AB, Crow JC, Perrett CW, Reid WM. Cell cycle proteins as molecular markers of malignant change in vulvar lichen sclerosus. IntJGynecolCancer. 2001;11(2):113-8.
13. Kokka F, Singh N, Faruqi A, Gibbon K, Rosenthal AN. Is differentiated vulval intraepithelial neoplasia the precursor lesion of human papillomavirus-negative vulval squamous cell carcinoma? IntJGynecolCancer. 2011;21(7):1297-305.
14. Gonzalez BJ, Magrina JF, Gaffey TA, Hernandez JL, Webb MJ, Cliby WA, et al. Long-term survival and disease recurrence in patients with primary squamous cell carcinoma of the vulva. GynecolOncol. 2005;97(3):828-33.

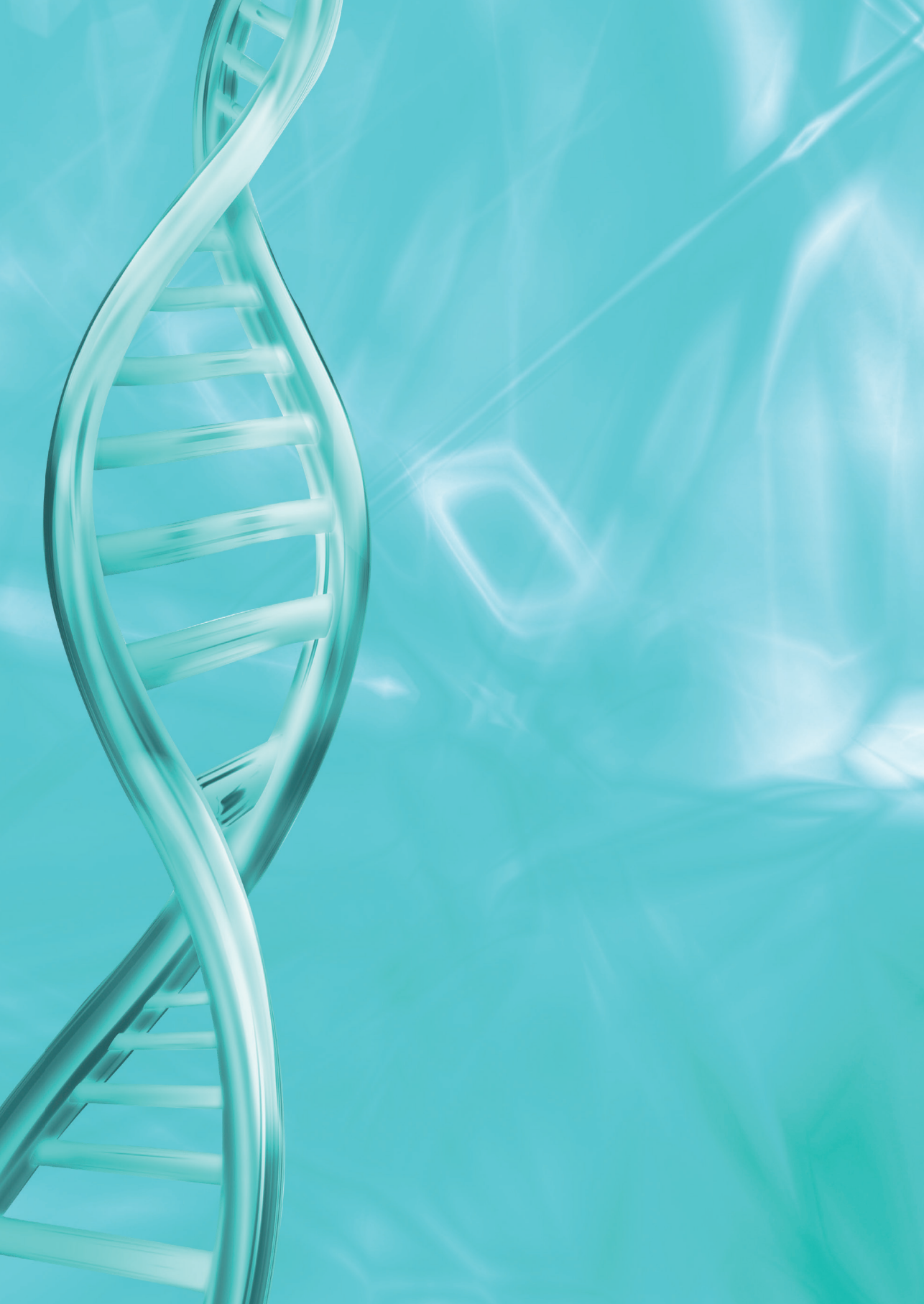
15. Coulter J, Gleeson N. Local and regional recurrence of vulval cancer: management dilemmas. Best Pract Res Clin Obstet Gynaecol. 2003;17(4):663-81.
16. Cormio G, Loizzi V, Carriero C, Cazzolla A, Putignano G, Selvaggi L. Groin recurrence in carcinoma of the vulva: management and outcome. EurJCancer Care (Engl). 2010;19(3):302-7.
17. Hopkins MP, Reid GC, Morley GW. The surgical management of recurrent squamous cell carcinoma of the vulva. Obstet Gynecol. 1990;75(6):1001-5.
18. Dutt A, Salvesen HB, Chen TH, Ramos AH, Onofrio RC, Hatton C, et al. Drug-sensitive FGFR2 mutations in endometrial carcinoma. ProcNatlAcadSciUSA. 2008;105(25):8713-7.
19. Gasche JA, Goel A. Epigenetic mechanisms in oral carcinogenesis. FutureOncol. 2012;8(11):1407-25.
20. McIntyre JB, Wu JS, Craighead PS, Phan T, Kobel M, Lees-Miller SP, et al. PIK3CA mutational status and overall survival in patients with cervical cancer treated with radical chemoradiotherapy. GynecolOncol. 2013;128(3):409-14.
21. Santarpia L, Qi Y, Stemke-Hale K, Wang B, Young EJ, Booser DJ, et al. Mutation profiling identifies numerous rare drug targets and distinct mutation patterns in different clinical subtypes of breast cancers. Breast Cancer ResTreat. 2012;134(1):333-43.
22. Wegman P, Ahlin C, Sorbe B. Genetic alterations in the K-Ras gene influence the prognosis in patients with cervical cancer treated by radiotherapy. IntJGynecolCancer. 2011;21(1):86-91.
23. Worsham MJ, Chen KM, Meduri V, Nygren AO, Errami A, Schouten JP, et al. Epigenetic events of disease progression in head and neck squamous cell carcinoma. ArchOtolaryngolHead Neck Surg. 2006;132(6):668-77.
24. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. NEnglJMed. 2011;364(26):2507-16.
25. Choschzick M, Hantaredja W, Tennstedt P, Gieseking F, Wolber L, Simon R. Role of TP53 mutations in vulvar carcinomas. Int J Gynecol Pathol. 2011;30(5):497-504.
26. Rolfe KJ, MacLean AB, Crow JC, Benjamin E, Reid WM, Perrett CW. TP53 mutations in vulval lichen sclerosus adjacent to squamous cell carcinoma of the vulva. BrJCancer. 2003;89(12):2249-53.
27. Holway AH, Rieger-Christ KM, Miner WR, Cain JW, Dugan JM, Pezza JA, et al. Somatic mutation of PTEN in vulvar cancer. ClinCancer Res. 2000;6(8):3228-35.
28. Soufir N, Queille S, Liboutet M, Thibaudeau O, Bachelier F, Delestaing G, et al. Inactivation of the CDKN2A and the p53 tumour suppressor genes in external genital carcinomas and their precursors. BrJDermatol. 2007;156(3):448-53.
29. Aide S, Lattario FR, Almeida G, do Val IC, Carvalho MG. Promoter hypermethylation of death-associated protein kinase and p16 genes in vulvar lichen sclerosus. JLow GenitTractDis. 2012;16(2):133-9.
30. Guerrero D, Guarch R, Ojer A, Casas JM, Mendez-Meca C, Esteller M, et al. Differential hypermethylation of genes in vulvar cancer and lichen sclerosus coexisting or not with vulvar cancer. IntJCancer. 2011;128(12):2853-64.
31. Kelemen LE, Kobel M, Chan A, Taghaddos S, Dinu I. Differentially Methylated Loci Distinguish Ovarian Carcinoma Histological Types: Evaluation of a DNA Methylation Assay in FFPE Tissue. BiomedResInt. 2013;2013:815894.

32. Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, et al. A unique regulatory phase of DNA methylation in the early mammalian embryo. Nature. 2012;484(7394):339-44.
33. Gasco M, Sullivan A, Repellin C, Brooks L, Farrell PJ, Tidy JA, et al. Coincident inactivation of 14-3-3sigma and p16INK4a is an early event in vulval squamous neoplasia. Oncogene. 2002;21(12):1876-81.
34. O'Nions J, Brooks LA, Sullivan A, Bell A, Dunne B, Rozycka M, et al. p73 is over-expressed in vulval cancer principally as the Delta 2 isoform. Br J Cancer. 2001;85(10):1551-6.
35. Gambichler T, Terras S, Kreuter A, Skrygan M. Altered global methylation and hydroxymethylation status in vulvar lichen sclerosus - further support for epigenetic mechanisms. Br J Dermatol. 2013.
36. Growdon WB, Boisvert SL, Akhavanfard S, Oliva E, Dias-Santagata DC, Kojiro S, et al. Decreased survival in EGFR gene amplified vulvar carcinoma. Gynecol Oncol. 2008;111(2):289-97.
37. Pilotti S, Donghi R, D'Amato L, Giarola M, Longoni A, Della TG, et al. HPV detection and p53 alteration in squamous cell verrucous malignancies of the lower genital tract. Diagn MolPathol. 1993;2(4):248-56.
38. Kurvinen K, Tervahauta A, Syrjanen S, Chang F, Syrjanen K. The state of the p53 gene in human papillomavirus (HPV)-positive and HPV-negative genital precancer lesions and carcinomas as determined by single-strand conformation polymorphism analysis and sequencing. Anticancer Res. 1994;14(1A):177-81.
39. Lee YY, Wilczynski SP, Chumakov A, Chih D, Koeffler HP. Carcinoma of the vulva: HPV and p53 mutations. Oncogene. 1994;9(6):1655-9.
40. Milde-Langosch K, Albrecht K, Joram S, Schlechte H, Giessing M, Loning T. Presence and persistence of HPV infection and p53 mutation in cancer of the cervix uteri and the vulva. Int J Cancer. 1995;63(5):639-45.
41. Pilotti S, D'Amato L, Della TG, Donghi R, Longoni A, Giarola M, et al. Papillomavirus, p53 alteration, and primary carcinoma of the vulva. Diagn MolPathol. 1995;4(4):239-48.
42. Kim YT, Thomas NF, Kessis TD, Wilkinson EJ, Hedrick L, Cho KR. p53 mutations and clonality in vulvar carcinomas and squamous hyperplasias: evidence suggesting that squamous hyperplasias do not serve as direct precursors of human papillomavirus-negative vulvar carcinomas. HumPathol. 1996;27(4):389-95.
43. Sliutz G, Schmidt W, Tempfer C, Speiser P, Gitsch G, Eder S, et al. Detection of p53 point mutations in primary human vulvar cancer by PCR and temperature gradient gel electrophoresis. Gynecol Oncol. 1997;64(1):93-8.
44. Wong YF, Chung TK, Cheung TH, Nobori T, Yim SF, Lai KW, et al. p16INK4 and p15INK4B alterations in primary gynecologic malignancy. Gynecol Oncol. 1997;65(2):319-24.
45. Flowers LC, Wistuba II, Scurry J, Muller CY, Ashfaq R, Miller DS, et al. Genetic changes during the multistage pathogenesis of human papillomavirus positive and negative vulvar carcinomas. J Soc Gynecol Investig. 1999;6(4):213-21.
46. Ngan HY, Cheung AN, Liu SS, Yip PS, Tsao SW. Abnormal expression or mutation of TP53 and HPV in vulvar cancer. EurJ Cancer. 1999;35(3):481-4.

47. Brooks LA, Tidy JA, Gusterson B, Hiller L, O'Nions J, Gasco M, et al. Preferential retention of codon 72 arginine p53 in squamous cell carcinomas of the vulva occurs in cancers positive and negative for human papillomavirus. Cancer Res. 2000;60(24):6875-7.
48. Marin MC, Jost CA, Brooks LA, Irwin MS, O'Nions J, Tidy JA, et al. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nature Genetics. 2000;25(1):47-54.
49. Wada H, Enomoto T, Yoshino K, Ozaki K, Kurachi H, Nomura T, et al. Immunohistochemical localization of telomerase hTERT protein and analysis of clonality in multifocal vulvar intraepithelial neoplasia. Am J Clin Pathol. 2000;114(3):371-9.
50. Rampone A, Vozza G, Vozza A, Rampone B, Capuano I, Tirabasso S, et al. Lichen and vulvar cancer. Personal experience. [Italian] I lichen ed il carcinoma vulvare. Nostra esperienza. Giornale Italiano di Dermatologia e Venereologia. 2002;137(4):267-9.
51. Reddy A, Yuille M, Sullivan A, Repellin C, Bell A, Tidy JA, et al. Analysis of CHK2 in vulval neoplasia. Br J Cancer. 2002;86(5):756-60.
52. Vanin K, Scurry J, Thorne H, Yuen K, Ramsay RG. Overexpression of wild-type p53 in lichen sclerosus adjacent to human papillomavirus-negative vulvar cancer. J Invest Dermatol. 2002;119(5):1027-33.
53. Almeida G, do V, I, Gondim C, Lima R, Takiya C, Carvalho G. Human papillomavirus, Epstein-Barr virus and p53 mutation in vulvar intraepithelial neoplasia. J ReprodMed. 2004;49(10):796-9.
54. Chulvis do Val IC, Almeida Filho GL, Valiante PM, Gondim C, Takiya CM, Carvalho MG. Vulvar intraepithelial neoplasia p53 expression, p53 gene mutation and HPV in recurrent/progressive cases. J ReprodMed. 2004;49(11):868-74.
55. Olawaiye A, Lee LM, Krasner C, Horowitz N. Treatment of squamous cell vulvar cancer with the anti-EGFR tyrosine kinase inhibitor Tarceva. Gynecologic Oncology. 2007;106(3):628-30.
56. Osakabe M, Hayashi M, Katayama Y, Emura I, Nemoto K, Umezu H, et al. Characteristics of vulvar squamous cell carcinoma in Japanese women. PatholInt. 2007;57(6):322-7.
57. Tapp RA, Feng J, Jones JW, Carlson JA, Wilson VL. Single base instability is promoted in vulvar lichen sclerosus. JInvest Dermatol. 2007;127(11):2563-76.
58. Aulmann S, Schleibaum J, Penzel R, Schirmacher P, Gebauer G, Sinn HP. Gains of chromosome region 3q26 in intraepithelial neoplasia and invasive squamous cell carcinoma of the vulva are frequent and independent of HPV status. JClinPathol. 2008;61(9):1034-7.
59. Janku F, Tsimberidou AM, Garrido-Laguna I, Wang X, Luthra R, Hong DS, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. Molecular Cancer Therapeutics. 2011;10(3):558-65.
60. Horowitz NS, Olawaiye AB, Borger DR, Growdon WB, Krasner CN, Matulonis UA, et al. Phase II trial of erlotinib in women with squamous cell carcinoma of the vulva. Gynecol Oncol. 2012;127(1):141-6.
61. Trietsch MD, Spaans VM, ter Haar NT, Osse EM, Peters AA, Gaarenstroom KN, et al. CDKN2A(p16) and HRAS are frequently mutated in vulvar squamous cell carcinoma. GynecolOncol. 2014;135(1):149-55.
62. Scheistroen M, Trope C, Pettersen EO, Nesland JM. p53 protein expression in squamous cell carcinoma of the vulva. Cancer. 1999;85(5):1133-8.

63. Bryndorf T, Kirchhoff M, Larsen J, Andreasson B, Bjerregaard B, Westh H, et al. The most common chromosome aberration detected by high-resolution comparative genomic hybridization in vulvar intraepithelial neoplasia is not seen in vulvar squamous cell carcinoma. CytogenetGenome Res. 2004;106(1):43-8.
64. Micci F, Panagopoulos I, Haugom L, Dahlback H-S, Pretorius ME, Davidson B, et al. Genomic aberration patterns and expression profiles of squamous cell carcinomas of the vulva. Genes Chromosomes and Cancer. 2013;52(6):551-63.
65. Lin MC, Mutter GL, Trivijisilp P, Boynton KA, Sun D, Crum CP. Patterns of allelic loss (LOH) in vulvar squamous carcinomas and adjacent noninvasive epithelia. AmJPathol. 1998;152(5):1313-8.
66. Pinto AP, Lin M-C, Mutter GL, Sun D, Villa LL, Crum CP. Allelic loss in human papillomavirus-positive and -negative vulvar squamous cell carcinomas. American Journal of Pathology. 1999;154(4):1009-15.
67. Carlson JA, Healy K, Tran TA, Malfetano J, Wilson VL, Rohwedder A, et al. Chromosome 17 aneusomy detected by fluorescence in situ hybridization in vulvar squamous cell carcinomas and synchronous vulvar skin. American Journal of Pathology. 2000;157(3):973-83.
68. Jee KJ, Kim YT, Kim KR, Kim HS, Yan A, Knuutila S. Loss in 3p and 4p and gain of 3q are concomitant aberrations in squamous cell carcinoma of the vulva. Modern Pathology. 2001;14(5):377-81.
69. Allen DG, Hutchins AM, Hammet F, White DJ, Scurry JP, Tabrizi SN, et al. Genetic aberrations detected by comparative genomic hybridisation in vulvar cancers. BrJCancer. 2002;86(6):924-8.
70. Rosenthal AN, Ryan A, Hopster D, Surentheran T, Jacobs IJ. High frequency of loss of heterozygosity in vulval intraepithelial neoplasia (VIN) is associated with invasive vulval squamous cell carcinoma (VSCC). IntJCancer. 2001;94(6):896-900.
71. Huang FY, Kwok YKY, Lau ET, Tang MHY, Ng TY, Ngan HYS. Genetic abnormalities and HPV status in cervical and vulvar squamous cell carcinomas. Cancer Genetics and Cytogenetics. 2005;157(1):42-8.
72. Yangling O, Shulang Z, Rongli C, Bo L, Lili C, Xin W. Genetic imbalance and human papillomavirus states in vulvar squamous cell carcinomas. European Journal of Gynaecological Oncology. 2007;28(6):442-6.
73. Lavorato-Rocha AM, De Melo MB, Rodrigues IS, Stiepcich MMA, Baiocchi G, Cestari FMDS, et al. Prognostication of vulvar cancer based on p14ARF status: Molecular assessment of transcript and protein. Annals of Surgical Oncology. 2013;20(1):31-9.
74. Bujko M, Kowalewska M, Zub R, Radziszewski J, Bidzinski M, Siedlecki JA. Lack of microsatellite instability in squamous cell vulvar carcinoma. Acta Obstet Gynecol Scand. 2012;91(3):391-4.
75. Pinto AP, Lin MC, Sheets EE, Muto MG, Sun D, Crum CP. Allelic imbalance in lichen sclerosus, hyperplasia, and intraepithelial neoplasia of the vulva. GynecolOncol. 2000;77(1):171-6.
76. Aide S, Lattario FR, Almeida G, do Val IC, Carvalho MG. Promoter hypermethylation patterns of death-associated protein kinase and p16 genes in vulvar lichen sclerosus. J Low GenitTract Dis. 2010;14(4):282-6.
77. Guerrero-Setas D, Perez-Janices N, Ojer A, Blanco-Fernandez L, Guarch-Troyas C, Guarch R. Differential gene hypermethylation in genital lichen sclerosus and cancer: a comparative study. Histopathology. 2013;63(5):659-69.

78. Lerma E, Esteller M, Herman JG, Prat J. Alterations of the p16/Rb/cyclin-D1 pathway in vulvar carcinoma, vulvar intraepithelial neoplasia, and lichen sclerosus. HumPathol. 2002;33(11):1120-5.
79. Oonk MH, Eijsink JJ, Volders HH, Hollema H, Wisman GB, Schuuring E, et al. Identification of inguinofemoral lymph node metastases by methylation markers in vulvar cancer. Gynecol Oncol. 2012;125(2):352-7.
80. Rosenthal AN, Hopster D, Ryan A, Jacobs IJ. Immunohistochemical analysis of p53 in vulval intraepithelial neoplasia and vulval squamous cell carcinoma. Br J Cancer. 2003;88(2):251-6.
81. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. NatMed. 2011;17(3):330-9.



CHAPTER 6

Stathmin is a highly sensitive and specific biomarker for vulvar high-grade squamous intraepithelial lesions

Linda S. Nooij
Enno J. Dreef
Vincent T.H.B.M. Smit
Mariëtte I.E. van Poelgeest
Tjalling Bosse

Journal of Clinical Pathology 2016;69:1070-1075

Abstract

Aims: Differentiating between HPV-dependent vulvar low-grade and high-grade squamous intraepithelial lesions (LSILs and HSILs) remains difficult in selected cases. Stathmin, a protein involved in cell cycle progression, might be a useful additional marker for this differentiation. The aim of this study is to investigate the additional diagnostic value of stathmin expression in vulvar intraepithelial neoplastic (VIN) lesions.

Methods: Immunohistochemical analysis was used to evaluate stathmin, P16 and Ki67 expression in 91 samples, including LSILs (n=16), HSILs (n=50), differentiated VIN (dVIN; n=10), lichen sclerosis (LS; n=10), and normal vulvar tissue (n=5).

Results: Stathmin was expressed in more than one-third of the epithelium in all HSILs and in 20% of LSILs. P16 and Ki67 were expressed in more than one-third of the epithelium in 94% of HSILs and in 13% and 40% of LSILs, respectively. Stathmin was expressed in more than one-third of the epithelium in 10% of the dVIN and in none of the LS or normal lesions. P16 and Ki67 expression was not present in more than one-third of the epithelium in any of these lesions. The sensitivity of stathmin for differentiating between LSILs and HSILs was 100% compared to a sensitivity of 94% for both p16 and Ki67. The specificity of stathmin, p16 and Ki67 was 80%, 87% and 60%, respectively.

Conclusions: Stathmin is a highly sensitive and specific biomarker for the diagnosis of vulvar HSIL. In addition to the more commonly used immunohistochemical markers p16 and Ki67, stathmin can be a useful diagnostic tool for identifying HSILs, especially in cases in which differentiating between LSIL and HSIL is difficult.

Introduction

Treatment of vulvar precursor lesions is a challenge for gynaecologists, and accurate differentiation between high-grade and low-grade vulvar precursor lesions is important for their clinical management (1). The nomenclature for vulvar lesions has changed in the last years. The most recent classification system of WHO (2014) and the International Society for the Study of Vulvar Disease (ISSVD, 2015) endorses a two-tiered system for human papilloma virus (HPV)-dependent intraepithelial lesions as low-grade squamous intraepithelial lesions (LSILs; flat condyloma, formerly termed vulvar intraepithelial neoplasia (VIN) 1) or high-grade squamous intraepithelial lesions (HSILs, formerly termed VIN2/3). Furthermore, this classification system discriminates between these HPV-dependent precursor lesions and the HPV-independent precursor lesion differentiated VIN (dVIN), which is associated with lichen sclerosis (LS) (2-5).

Vulvar LSILs encompass a range of HPV-associated vulvar lesions that are not pre-cancerous and do not require treatment unless they are symptomatic. In 90% of the vulvar LSILs, the associated HPV types are HPV 6 and 11 (6-8). Treatment can consist of the application of immunomodulating cream, podophyllin, cryotherapy, laser therapy or surgery (8-10). Vulvar HSIL is associated with high-risk HPV types, namely types 16 and 18, and has a 9%–16% chance of progression to vulvar squamous cell carcinoma (VSCC), if left untreated. The HPV-independent precursor lesion dVIN is an uncommon vulvar lesion that has been recognised as a distinctive diagnosis since the mid-1980s. The subtle clinical and histological changes make recognition and diagnosis difficult, which might contribute to the low prevalence (5,11,12). Importantly, the malignant potential of untreated dVIN lesions is probably as high as 80% (9,12,13). Given the malignant potential of HSILs and dVIN, it is important to treat these patients adequately and to ensure close follow-up. In addition, vulvar HSILs are often multifocal and are sometimes associated with cervical and vaginal intraepithelial neoplasia (13). For these reasons, it is clinically important to have an accurate histopathological diagnosis and to reliably distinguish between LSILs, HSILs and dVIN (1). Tangential sectioning, small biopsies, thermal artefacts, coexistent inflammatory or reactive epithelial atypia (with or without LS) and the application of subjective criteria all contribute to the difficulty of VIN diagnosis and grading (10,13). Two studies have investigated interobserver variability between LSIL and HSIL vulvar lesions and found moderate-to-good agreement of 73.9% and 82%, respectively (1,14). Experienced gynaecological pathologists show good agreement (67%) in distinguishing HPV-dependent from HPV-independent vulvar lesions (15), but the histopathological diagnosis of dVIN is more difficult, and the interobserver and intraobserver variability is high (11).

Currently, immunohistochemical staining of p16, p53 and Ki67 is widely used for the differential diagnosis of vulvar precursor lesions. P16, a cyclin-dependent kinase-4 inhibitor, is especially useful for differentiating between HPV-dependent VIN (p16-positive) and dVIN (p16-negative). The E6 and E7 proteins of oncogenic HPV bind and inactivate p53 and pRb, leading to unregulated cell proliferation. This results in compensatory expression of the p16 tumour suppressor protein; thus, immunohistochemical staining of p16 is an accurate marker for HPV (10,11,13,16-18). However, p16 staining can be less specific for differentiating between vulvar LSIL and HSIL lesions, since these lesions sometimes show similar p16 expression patterns. Furthermore, the p16 staining pattern is sometimes difficult to interpret due to differential staining intensity and patterns that can also be found in inflammatory vulvar disorders (9,13,19-21). Ki67, a cell proliferation marker, is widely used to differentiate between cervical LSILs and HSILs (9,13,20,22). Several studies have shown that increased expression of Ki67 is associated with higher cervical SIL grade. In particular, the sensitivity of Ki67 in detecting cervical HSIL is high (93%–95%) (20,22-24). Because of the similarities between cervical intraepithelial neoplasia and VIN lesions, Ki67 has become a commonly used marker for VIN lesions as well, (7,14) and Ki67 staining is useful for differentiating between dVIN and normal vulvar epithelium (9,11,13,14,25). In dVIN, Ki67 positivity is usually confined to the basal layers of the epithelium, while in normal vulvar epithelium, Ki67 staining is completely negative (11,13,14,25). Notably, few studies have investigated Ki67 expression in vulvar SILs (7,13,20).

In contrast to HPV-dependent VSCC and vulvar HSIL, the tumour suppressor gene *TP53* is frequently mutated in HPV-negative VSCC and in its precursor lesion, dVIN. Immunohistochemical staining of p53 can thus be used as a marker for discriminating between HPV-independent and HPV-dependent precursors. A mutation in *TP53* can result in one of two patterns of aberrant expression on immunohistochemical staining that is, either strong diffuse p53 staining or a complete absence of staining (17,26). Despite the value of these widely-used markers, there are cases in which differentiation is difficult, and p16, Ki67 and p53 staining do not give a definite diagnosis.

Stathmin-1, which this study refers to as stathmin, is a ubiquitous microtubule-destabilising phosphoprotein in humans that is involved in cell cycle progression (27,28). Stathmin regulates microtubule dynamics and is required for all cellular processes that involve microtubule rearrangement, mainly mitosis. Accordingly, stathmin activity is critically important for cell division (29,30). Stathmin has been postulated to be an immunohistochemical marker for differentiating between low-grade and high-grade intra-epithelial diseases (28,29,31). One study showed that stathmin staining had greater specificity (93%) than p16 staining (44%) for detecting cervical HSILs. Stathmin staining distinguished HSILs from the majority of LSIL precursors (28).

Another study investigated stathmin as a marker of early neoplasia in the fallopian tube and found that stathmin could discriminate between normal fallopian tube epithelium, tubal intraepithelial carcinoma and invasive serous carcinoma (29).
In this study, we investigated stathmin expression in normal vulvar mucosa, vulvar LSILs and HSILs, dVIN and LS to determine whether stathmin can serve as an additional marker for the diagnosis of vulvar HSIL. In addition, we investigated whether stathmin could discriminate between HPV-dependent and HPV-independent precursor lesions.

Materials and methods

Cases

A total of 86 vulvar samples (resection, *n*=38; biopsy, *n*=48) were obtained from the surgical pathology archives of the Leiden University Medical Center after approval by the institutional review board. The samples included vulvar LSILs (originally reported as VIN 1 or condylomata lesions, but referred to as LSILs in this study; *n*=15); HSILs (originally reported as VIN 2/3 lesions, but referred to as HSILs in this study; *n*=51); dVIN (*n*=10); or LS (*n*=10). In addition, we analysed five normal vulvar epithelium samples from patients who underwent labia reduction surgery and who gave permission for the use of the material for research purposes. An overview of the classification of the patient samples is given in table 1. H&E stained slides were re-reviewed by a gynaecological pathologist (TB), and the diagnosis was confirmed in 81 (95%) cases. In four cases, the initial diagnosis was adjusted. One LSIL was reclassified as a HSIL, and three HSILs were reclassified as LSILs. The revised diagnoses were used in the final analysis. The classification of the vulvar lesions was performed according to the criteria described in the WHO and ISSVD classification systems (2,3).

Table 1: Sample characteristics

	Total samples N= 91
LSIL	15 (16,5%)
HSIL	51 (56%)
dVIN	10 (11%)
Lichen Sclerosis	10 (11%)
Normal vulvar epithelium	5 (5,5%)

LSIL: Low grade squamous intraepithelial lesion
HSIL: High grade squamous intraepithelial lesion
dVIN: Differentiated vulvar intraepithelial neoplasia

Immunohistochemistry

All samples were evaluated for stathmin, p16 and Ki67 expression by immunohistochemistry, and the HPV-independent dVIN and LS samples were also evaluated for p53 expression. The HPV-dependent samples were not stained for p53, because of the expected wildtype expression pattern in these lesions (17,32,33). Serial sections of 4-µm thickness were cut from formalin-fixed paraffin-embedded specimen blocks and dried overnight at 37°C. The tissue sections were deparaffinised, rehydrated and incubated in 0.3% hydrogen peroxidase (H_2O_2) solution for 20 min to block endogenous peroxidase activity. Antigen retrieval was carried out by microwave treatment in 0.01 M citrate buffer (pH 6.0) for 12 min. Slides were incubated overnight at room temperature with a polyclonal rabbit antibody to stathmin (Cell Signaling Technology, Danvers, MA, USA; 1:50 dilution; clone # 3352), a monoclonal mouse antibody to p16 (M Tm Laboratories, Westborough, MA, USA; 1:50 dilution; clone E6H4), a monoclonal mouse antibody to Ki67 (Dako, Denmark; 1:100 dilution; clone MIB-1) and a monoclonal mouse antibody to p53 (Thermo Scientific, 1:2000 dilution; clone DO-7) diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing with PBS, tissue sections were incubated with PowerVision-Poly/HRP (Immunologic, The Netherlands) for 30 min. Immunoreactions were visualised using 0.5% 3.3'-diamino-benzidine-tetrahydrochloride and 0.02% H_2O_2 in Tris-HCl. The sections were then counterstained with haematoxylin. Because many samples contained both lesional and non-lesional areas, we considered the non-lesional areas as internal controls. Furthermore, immunohistochemical stainings were performed in series and the study sets included at least some positive and negative cases. Therefore, we did not add an external positive and negative control sample.

Evaluation of stathmin, p16, Ki67 and p53 expression

Two independent observers (LSN and TB) scored the immunohistochemical patterns, and consensus was reached by discussing cases with discordant initial scores. Stathmin staining was scored as 0 (all cells negative), 1+ (positive staining in less than one-third of the epithelial thickness), 2+ (positive staining in one-third to two-thirds of the epithelial thickness) or 3+ (positive staining in more than two-thirds of the epithelial thickness). Because there is not yet a validated cut-off value for interpretation of stathmin staining, first we evaluated the stathmin staining patterns in five normal and five dysplastic vulvar lesions. We found that stathmin expression was sometimes present in the basal layers of normal vulvar epithelium, but it was not present in more than one-third of the epithelial thickness. Therefore, similar to the interpretation of Ki67 expression, we decided to use stathmin expression in more than one-third of the epithelium as the cut-off value for increased expression (13,16,28). For the statistical analysis and determination of specificity and sensitivity, the staining results were subdivided into two groups: cytoplasmic or nuclear immunoreactivity in less than one-third of the epithelial

thickness (all samples that were scored 0 and 1+) or more than one-third of the epithelial thickness (all samples that were scored 2+ and 3+). Immunostaining with p16 was considered positive when there was diffuse staining of epithelial cells (nuclear and/or cytoplasmatic) in more than one-third of the epithelium (28,34). Immunostaining with Ki67 was scored as expression present in more than one-third of the epithelium or in less than one-third of the epithelium (24). Immunostaining of p53 was scored as wild type (patchy basal positivity) or as an aberrant staining pattern (either a strong diffuse expression pattern when >25% of the cells showed strong positive nuclear staining, or a complete absence of staining) (17,26).

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics V.20.0; chi-squared tests were used to differentiate between HSIL and LSIL. The sensitivity and specificity of stathmin, p16 and Ki67 staining were calculated for diagnosing HSIL. In addition, the positive predictive value (PPV) and negative predictive value (NPV) of all of the immunohistochemical markers was determined.

Results

The immunohistochemical staining results for stathmin, p16 and Ki67 are summarised in table 2. Figure 1 shows examples of the staining results of samples categorised as LSIL, HSIL, dVIN and LS. The expression of stathmin was evaluated in the epithelial layers as well as in the stromal component. In the stromal component, we observed some positive staining in the immune infiltrate. Stathmin expression was completely absent in four (80%) of the normal vulvar epithelium samples. In one (20%) normal vulva sample, stathmin expression was present in less than one-third of the epithelium.

Table 2: Immunohistochemical results; the number of samples that were scored as expression in > 1/3th of the epithelium for stathmin, p16 and Ki67

Diagnosis	Stathmin	p16	Ki67
LSIL	3/15 (20%)	2/15 (13,3%)	6/15 (40%)
HSIL	51/51 (100%)	48/51 (94,1%)	48/51 (94,1%)
dVIN	1/10 (10%)	0/10 (0%)	0/10 (0%)
Lichen Sclerosis	0/10 (0%)	0/10 (0%)	0/10 (0%)
Normal vulvar epithelium	0/5 (0%)	0/5 (0%)	0/5 (0%)

LSIL: Low grade squamous intraepithelial lesion
HSIL: High grade squamous intraepithelial lesion
dVIN: Differentiated vulvar intraepithelial neoplasia

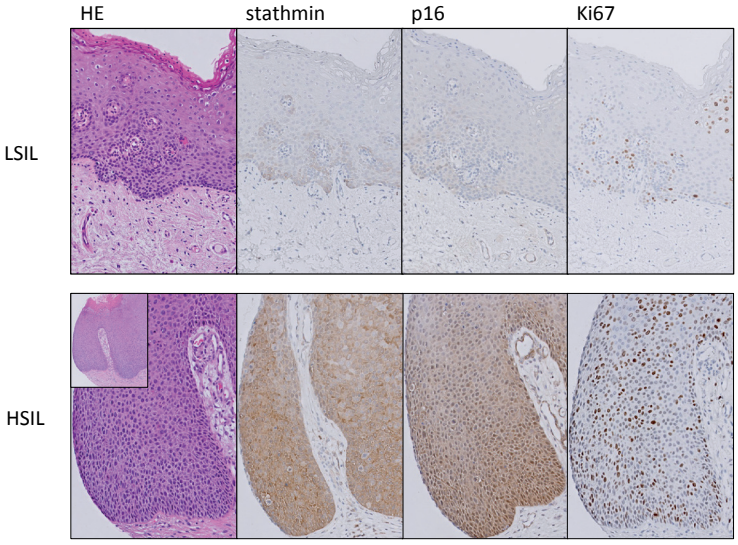


Figure 1a: H&E, stathmin, p16 and Ki67 staining patterns in a vulvar LSIL and HSIL. Inserted figure shows the HE of the HSIL on a lower magnification

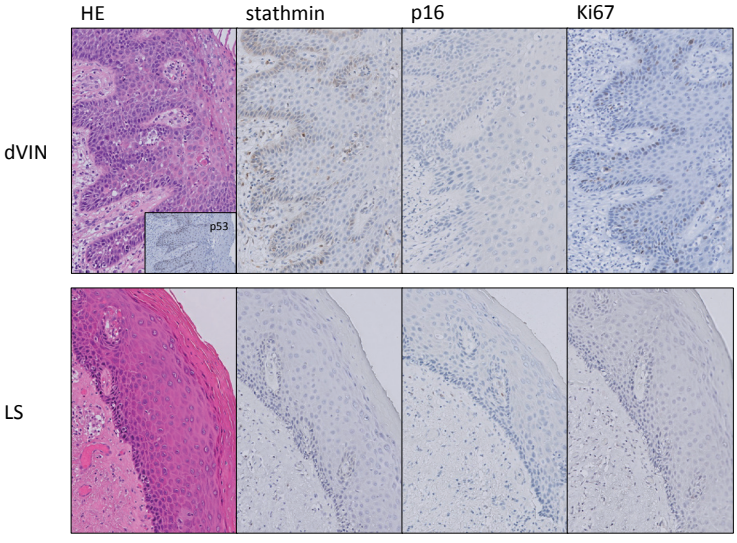


Figure 1b: H&E, stathmin, p16 and Ki67 staining patterns in a differentiated VIN (dVIN) and lichen sclerosus (LS) lesion. p53 staining in the dVIN sample showed a strong diffuse expression pattern, suggestive for a mutation in TP53 (inserted figure)

All of the HSILs showed stathmin expression in more than one-third of the epithelium. In 12 (80%) of the 15 LSILs, stathmin expression was confined to the basal layer of the epithelium (scored as 1+). The other three (20%) LSILs showed stathmin expression in more than one-third of the epithelium. Stathmin expression was completely absent (scored as 0) in four (40%) of the dVIN and seven (70%) of the LS lesions, and five (50%) of the dVIN and three (30%) of the LS lesions expressed stathmin in less than one-third of the epithelium. One (10%) dVIN sample showed stathmin expression in more than one-third of the epithelium. Staining with p16 was positive in more than one-third of the epithelium in 48 (94%) HSILs, and 2 (13%) of the LSILs also showed positive p16 staining in more than one-third of the epithelium. As expected, and in line with the initial diagnosis, all dVIN were completely negative for p16 expression (scored as 0). Seven (70%) of the 10 LS samples were completely negative for p16 staining, while the remaining 3 (30%) showed positivity in the basal keratinocytes (less than one-third of the epithelium). All normal vulvar epithelium samples were completely negative for p16 staining. Ki67 staining was present in more than one-third of the epithelium in 48 (94%) of the HSILs and in 6 (40%) of the LSILs. All other HSILs and LSILs showed Ki67 expression in the basal layer of the epithelium. All dVIN, LS and normal vulvar epithelium samples were scored as showing Ki67 expression in less than one-third of the epithelium. Of these samples, two (20%) of the dVIN and eight (80%) of the LS were completely negative for Ki67 staining.

Table 3 gives an overview of the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of stathmin, p16 and Ki67 for detecting vulvar HSILs. Stathmin showed higher sensitivity (100%) than p16 (94%) and Ki67 (94%). The specificity was lower than the sensitivity for all immunohistochemical markers: 80% for stathmin, and 87% and 60% for p16 and Ki67, respectively. The PPV was comparable for all markers, while the NPV was especially high for stathmin (100%) compared with p16 and Ki67 (NPVs of 81% and 75%, respectively).

Table 3: Sensitivity, specificity, positive predictive value and negative predictive value for differentiation between low- and high-grade vulvar squamous intraepithelial lesions

	Stathmin	p16	Ki67
Sensitivity (%)	100%	94%	94%
Specificity (%)	80%	87%	60%
PPV (%)	94%	96%	89%
NPV (%)	100%	81%	75%

PPV: positive predictive value
NPV: negative predictive value

We also evaluated the association between stathmin expression and p53 scoring in the HPV-independent dVIN and LS samples (Table 4). Seven (70%) of the dVIN samples were independent lesions and three (30%) adjacent to invasive cancer. Six of the seven independent dVIN lesions progressed towards invasive carcinoma during follow-up. In the dVIN cases, two (20%) were scored as having wild-type p53 expression (both were independent dVIN lesions, one with and one without progression towards VSCC), and the other samples (80%) showed an aberrant p53 staining pattern (either a strong diffuse expression pattern or no expression). In the LS cases, eight (80%) were scored having as wild type p53 expression, while the other two cases were scored as having aberrant p53 staining. All of the samples with p53 wild-type staining (*n*=10) showed stathmin expression in less than one-third of the epithelium. One of the dVIN cases with an aberrant p53 staining pattern showed stathmin expression in more than one-third of the epithelium.

Table 4: Association between stathmin expression and p53 staining in 20 HPV-independent vulvar precursor lesions (10 dVIN and 10 LS)

Stathmin	p53 wild type staining pattern	p53 aberrant staining pattern*
Expression in < 1/3th of the epithelium	10/10	9/10
Expression in > 1/3th of the epithelium	0/10	1/10

*Either highly expressed or completely absent P53 immunohistochemical staining indicating a possible P53 mutation

HPV: human papilloma virus
dVIN: differentiated vulvar intraepithelial neoplasia
LS: lichen sclerosis

Discussion

The aim of this study was to determine whether stathmin expression as measured by semiquantitative immunohistochemistry could improve the diagnosis and correct grading of vulvar SILs. Our findings indicate that stathmin is a highly sensitive and specific biomarker for the differentiation of vulvar LSILs and HSILs. The excellent sensitivity of stathmin (100%) exceeded that of the commonly used markers p16 and Ki67 (94% sensitivity for both). The specificity of stathmin was similar to that of p16 and exceeded that of Ki67.

In the most recent classification of vulvar squamous intraepithelial lesions the former VIN1 or flat condyloma has been adjusted towards LSIL, while the former VIN 2 and 3 or usual type VIN has been adjusted towards HSIL. In the new classification system,

dVIN remains a distinct entity (3,5). It is clinically important to differentiate between vulvar LSIL and HSIL, especially because of the malignant potential of vulvar HSIL, which is 9%–16% for untreated patients and 3% for patients who receive treatment. Spontaneous regression occurs in less than 1.5% of patients with vulvar HSIL patients, and it mostly occurs during the first 10 months following the diagnosis (35). In contrast, LSIL has a negligible chance of progression towards invasive VSCC (9,12,13). In view of these risks, it is clear that an adequate treatment plan is needed, especially for vulvar HSIL; current therapies include local treatment with the immunomodulating agent imiquimod, laser excision or surgery (9,12,13).
The commonly used immunohistochemical marker p16 is not always sufficient to differentiate between vulvar LSIL and HSIL (9,13). Additional use of Ki67 as a marker can help in this differentiation (14,25), but in some cases, doubt remains about the definite diagnosis. Our data show that stathmin expression in HPV-dependent vulvar dysplasia may be informative in cases in which there is doubt about the grading because of the high specificity and sensitivity of stathmin expression in more than one-third of the epithelium. After revision by an expert gynaecology pathologist, one case was upgraded from an LSIL to a HSIL, and in three cases, the initial diagnosis was revised from an HSIL to a LSIL. Interestingly, the Ki67 staining pattern was especially difficult to interpret in these three cases. Specifically, Ki67 staining was present in the parabasal layer, but it was also present in the upper epithelium, where koilocytic atypia is present, and this can easily be mistaken for an HSIL vulvar lesion. This Ki67 staining pattern has been described previously, but it is not well-known (6,7). Stathmin expression was negative in these samples, clearly demonstrating that stathmin staining is truly different from Ki67 staining.

Currently, there is no consensus on how to interpret the immunohistochemical staining results of stathmin expression. One study that looked at stathmin expression in cervical SIL defined increased stathmin expression as positive staining in more than two-thirds of the cervical epithelium (28). In contrast to that study, we used a cut-off of stathmin expression in more than one-third of the epithelium based on our preliminary evaluation of stathmin expression in normal vulvar epithelium and dysplastic lesions and based on our specificity and sensitivity results. When we used more than two-thirds of the epithelium as a cut-off value, the sensitivity decreased to 76% and the specificity increased slightly to 81%.

Morphological diagnosis of especially dVIN is difficult and interobserver variability is high (11). dVIN and LS are frequently associated with mutation in the *TP53* gene (12). Therefore, staining with p53 can be helpful to differentiate between dVIN or LS and vulvar SILs, although p53 is not necessarily a marker for dVIN (36). One of our samples diagnosed as dVIN showed a p53 wild-type expression pattern and did not progress

towards an invasive tumour. Consequently, it can be possible that this sample is not a genuine dVIN lesion. Previous studies have shown that there is a relationship between mutant p53 expression and increased stathmin expression in precancerous lesions (serous tubal intra-epithelial carcinomas (STICs)) of the fallopian tube (29,31). In these STICs, a *TP53* mutation results in upregulation of stathmin expression. The majority of *TP53* mutations result in loss of function. However, mutations in the *TP53* gene can also lead to a novel protein with a gain-of-function. Hypothetically, stathmin upregulation is needed to support this gain-of-function mutant p53 (29,31,37). Therefore, we evaluated the association of the p53 expression pattern with the stathmin expression pattern in HPV-independent vulvar precursors. Intriguingly, and in contrast with the association reported in STICs, we observed no relationship between immunohistochemical p53 and stathmin staining in HPV-independent vulvar precursors. This can be interpreted as arguing against a direct mechanistic link between *TP53* mutation and stathmin expression; at the very least, it shows that these two markers are unrelated in dVIN. Additional mechanistic studies are needed to gain a better understanding of this observation.

We are the first to describe stathmin staining in vulvar samples and this comes with some limitations. Although vulvar precursor lesions are uncommon, this was a relatively small study. Another limitation is the absence of a predetermined and validated cut-off value for increased stathmin expression. The cut-off value used in this study must be validated in an independent set of vulvar samples. Furthermore, we focused on the expression of stathmin in vulvar precursor lesions and therefore did not include VSCCs. Due to this focused approach, we are not informed about the expression and potential diagnostic utility of stathmin expression in vulvar carcinomas.

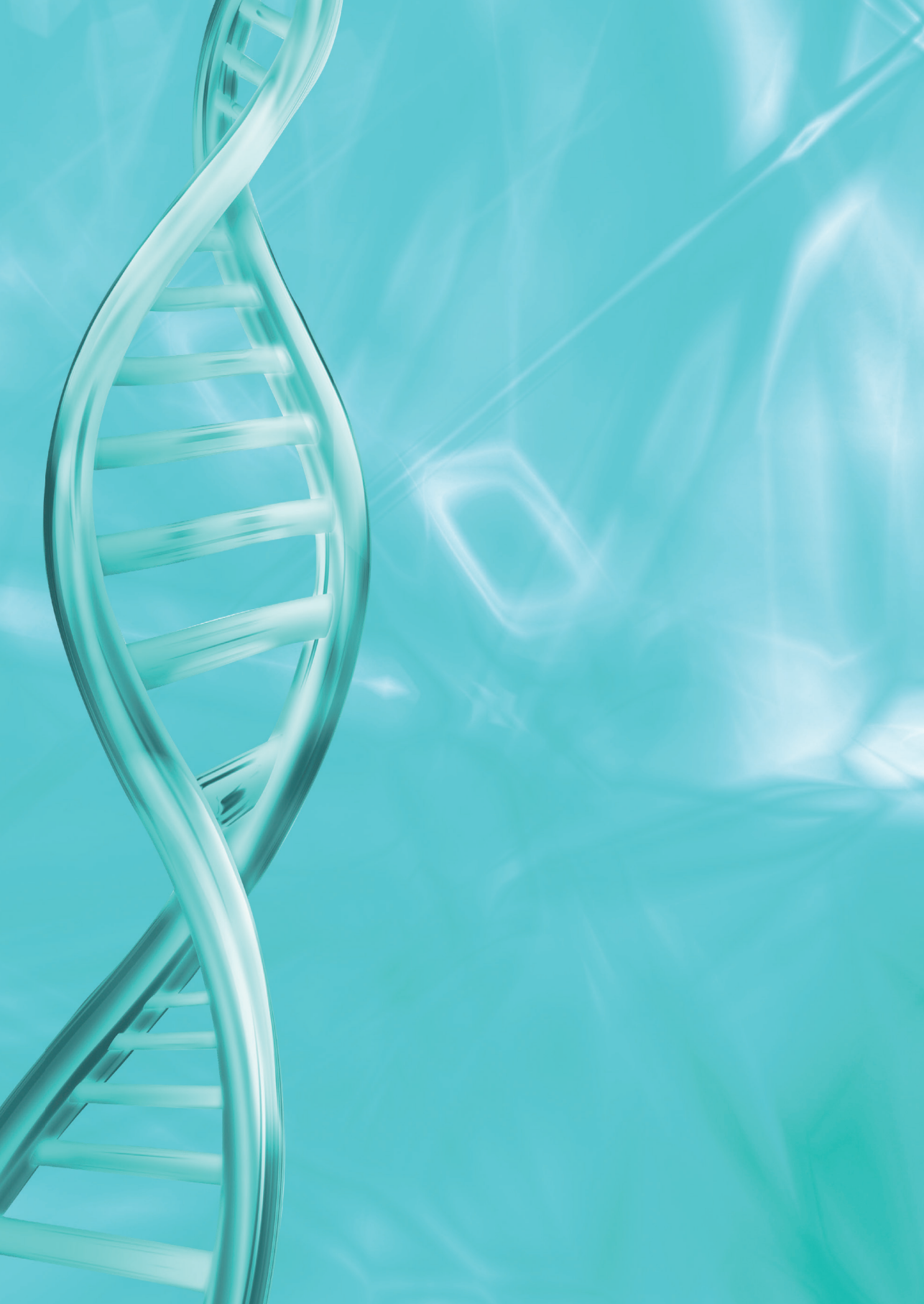
In conclusion, stathmin is a highly sensitive and specific biomarker for high-grade dysplasia of HPV-associated vulvar precursors. It can be used in addition to p16 and Ki67 staining in formalin-fixed, paraffin-embedded tissue sections using routine immunohistochemical procedures when differentiation between vulvar LSILs and HSILs is difficult. Before implementing stathmin in daily practice, validation in an independent cohort is necessary.

References

1. Preti M, Mezzetti M, Robertson C, Sideri M. Inter-observer variation in histopathological diagnosis and grading of vulvar intraepithelial neoplasia: results of an European collaborative study. *BJOG.* 2000;107(5):594-599.
2. Kurman RJ, Carcangiu ML, Herrington CS, Young RH. *WHO classification of Tumours of Female Reproductive Organs.* Lyon: IARC; 2014.
3. Sideri M, Jones RW, Wilkinson EJ, et al. Squamous vulvar intraepithelial neoplasia: 2004 modified terminology, ISSVD Vulvar Oncology Subcommittee. *J.Reprod.Med.* 2005;50(11):807-810.
4. Darragh TM, Colgan TJ, Thomas CJ, et al. The Lower Anogenital Squamous Terminology Standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Int.J.Gynecol.Pathol.* 2013;32(1):76-115.
5. Bornstein J. The 2015 Internation Society for the Study of Vulvovaginal Disease Terminology of Vulvar Squamous Intraepithelial Lesions. 2015.
6. Bai H, Cviko A, Granter S, Yuan L, Betensky RA, Crum CP. Immunophenotypic and viral (human papillomavirus) correlates of vulvar seborrheic keratosis. *Hum.Pathol.* 2003;34(6):559-564.
7. Logani S, Lu D, Quint WG, Ellenson LH, Pirog EC. Low-grade vulvar and vaginal intraepithelial neoplasia: correlation of histologic features with human papillomavirus DNA detection and MIB-1 immunostaining. *Mod.Pathol.* 2003;16(8):735-741.
8. Nelson EL, Bogliatto F, Stockdale CK. Vulvar Intraepithelial Neoplasia (VIN) and Condylomata. *Clin.Obstet.Gynecol.* 2015;58(3):512-525.
9. Leonard B, Kridelka F, Delbecque K, et al. A clinical and pathological overview of vulvar condyloma acuminatum, intraepithelial neoplasia, and squamous cell carcinoma. *Biomed.Res.Int.* 2014;2014:480573.
10. Preti M, Scurry J, Marchitelli CE, Micheletti L. Vulvar intraepithelial neoplasia. *Best.Pract.Res.Clin. Obstet.Gynaecol.* 2014;28(7):1051-1062.
11. van den Einden LC, de Hullu JA, Massuger LF, et al. Interobserver variability and the effect of education in the histopathological diagnosis of differentiated vulvar intraepithelial neoplasia. *Mod. Pathol.* 2013;26(6):874-880.
12. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. *Crit Rev.Oncol.Hematol.* 2008;68(2):131-156.
13. McCluggage WG. Premalignant lesions of the lower female genital tract: cervix, vagina and vulva. *Pathology.* 2013;45(3):214-228.
14. van Beurden M, de Craen AJ, de Vet HC, et al. The contribution of MIB 1 in the accurate grading of vulvar intraepithelial neoplasia. *J.Clin.Pathol.* 1999;52(11):820-824.
15. Trimble CL, Diener-West M, Wilkinson EJ, Zaino RJ, Kurman RJ, Shah KV. Reproducibility of the histopathological classification of vulvar squamous carcinoma and intraepithelial neoplasia. *J.Low Genit.Tract.Dis.* 1999;3(2):98-103.

16. O'Neill CJ, McCluggage WG. p16 expression in the female genital tract and its value in diagnosis. *Adv.Anat.Pathol.* 2006;13(1):8-15.
17. Santos M, Montagut C, Mellado B, et al. Immunohistochemical staining for p16 and p53 in premalignant and malignant epithelial lesions of the vulva. *Int.J.Gynecol.Pathol.* 2004;23(3):206-214.
18. Cheng AS, Karnezis AN, Jordan S, Singh N, McAlpine JN, Gilks CB. p16 Immunostaining Allows for Accurate Subclassification of Vulvar Squamous Cell Carcinoma Into HPV-Associated and HPV-Independent Cases. *Int.J.Gynecol.Pathol.* 2015.
19. Nogueira MC, Guedes Neto EP, Rosa MW, Zettler E, Zettler CG. Immunohistochemical expression of p16 and p53 in vulvar intraepithelial neoplasia and squamous cell carcinoma of the vulva. *Pathol. Oncol.Res.* 2006;12(3):153-157.
20. Pinto AP, Schlecht NF, Woo TY, Crum CP, Cibas ES. Biomarker (ProEx C, p16(INK4A), and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. *Mod.Pathol.* 2008;21(9):1067-1074.
21. Riethdorf S, Neffen EF, Cviko A, Loning T, Crum CP, Riethdorf L. p16INK4A expression as biomarker for HPV 16-related vulvar neoplasias. *Hum.Pathol.* 2004;35(12):1477-1483.
22. Sari AF, Safaei A, Pourjabali M, Momtahan M. Evaluation of Ki67, p16 and CK17 Markers in Differentiating Cervical Intraepithelial Neoplasia and Benign Lesions. *Iran J.Med.Sci.* 2013;38(1):15-21.
23. Kruse AJ, Baak JP, de Bruin PC, et al. Ki-67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading. *J.Pathol.* 2001;193(1):48-54.
24. Bulten J, van der Laak JA, Gemmink JH, Pahlplatz MM, de Wilde PC, Hanselaar AG. MIB1, a promising marker for the classification of cervical intraepithelial neoplasia. *J.Pathol.* 1996;178(3):268-273.
25. van der Avoort IA, van der Laak JA, Paffen A, et al. MIB1 expression in basal cell layer: a diagnostic tool to identify premalignancies of the vulva. *Mod.Pathol.* 2007;20(7):770-778.
26. Singh N, Leen SL, Han G, et al. Expanding the morphologic spectrum of differentiated VIN (dVIN) through detailed mapping of cases with p53 loss. *Am.J.Surg.Pathol.* 2015;39(1):52-60.
27. Belletti B, Baldassarre G. Stathmin: a protein with many tasks. New biomarker and potential target in cancer. *Expert Opin Ther Targets.* 2011;15(11):1249-1266.
28. Howitt BE, Nucci MR, Drapkin R, Crum CP, Hirsch MS. Stathmin-1 expression as a complement to p16 helps identify high-grade cervical intraepithelial neoplasia with increased specificity. *Am J Surg Pathol.* 2013;37(1):89-97.
29. Karst AM, Levanon K, Duraisamy S, et al. Stathmin 1, a marker of PI3K pathway activation and regulator of microtubule dynamics, is expressed in early pelvic serous carcinomas. *Gynecol Oncol.* 2011;123(1):5-12.
30. Baquero MT, Hanna JA, Neumeister V, et al. Stathmin expression and its relationship to microtubule-associated protein tau and outcome in breast cancer. *Cancer.* 2012;118(19):4660-4669.
31. Novak M, Lester J, Karst AM, et al. Stathmin 1 and p16 are sensitive adjunct biomarkers for serous tubal intraepithelial carcinoma. *Gynecol.Oncol.* 2015.

32. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. *Histopathology.* 2013;62(1):161-175.
33. Kohlberger PD, Kirnbauer R, Bancher D, et al. Absence of p53 protein overexpression in precancerous lesions of the vulva. *Cancer.* 1998;82(2):323-327.
34. Hampl M, Wentzensen N, Vinokurova S, et al. Comprehensive analysis of 130 multicentric intraepithelial female lower genital tract lesions by HPV typing and p16 expression profile. *J.Cancer Res.Clin.Oncol.* 2007;133(4):235-245.
35. van Seters M, van Beurden M, de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. *Gynecol. Oncol.* 2005;97(2):645-651.
36. Liegl B, Regauer S. p53 immunostaining in lichen sclerosus is related to ischaemic stress and is not a marker of differentiated vulvar intraepithelial neoplasia (d-VIN). *Histopathology.* 2006;48(3):268-274.
37. Sonego M, Schiappacassi M, Lovisa S, et al. Stathmin regulates mutant p53 stability and transcriptional activity in ovarian cancer. *EMBO Mol.Med.* 2013;5(5):707-722.



CHAPTER 7

Genomic characterisation of vulvar (pre)cancers identifies distinct molecular subtypes with prognostic significance

Linda S. Nooij
Natalja T. ter Haar
Dina Ruano
Natalia Rakislova
Tom van Wezel
Vincent T.H.B.M. Smit
Baptist J.B.M.Z. Trimbos
Jaume Ordi
Mariette I.E. van Poelgeest
Tjalling Bosse

Clinical Cancer Research 2017;23:6781-6789

Abstract

Purpose: Vulvar cancer (VC) can be subclassified by human papillomavirus (HPV) status. HPV-negative VCs frequently harbor *TP53* mutations; however, in-depth analysis of other potential molecular genetic alterations is lacking. We comprehensively assessed somatic mutations in a large series of vulvar (pre)cancers.

Experimental Design: We performed targeted next-generation sequencing (17 genes), p53 immunohistochemistry and HPV testing on 36 VC and 82 precursors (sequencing cohort). Subsequently, the prognostic significance of the three subtypes identified in the sequencing cohort was assessed in a series of 236 VC patients (follow-up cohort).

Results: Frequent recurrent mutations were identified in HPV-negative vulvar (pre) cancers in *TP53* (42% and 68%), *NOTCH1* (28% and 41%), and *HRAS* (20% and 31%). Mutation frequency in HPV-positive vulvar (pre)cancers was significantly lower (P=0.001). Furthermore, a substantial subset of the HPV-negative precursors (35/60, 58.3%) and VC (10/29, 34.5%) were *TP53* wild-type (wt), suggesting a third, not-previously described, molecular subtype. Clinical outcomes in the three different subtypes (HPV+, HPV-/p53wt, HPV-/p53abn) were evaluated in a follow-up cohort consisting of 236 VC patients. Local recurrence rate was 5.3% for HPV+, 16.3% for HPV-/p53wt and 22.6% for HPV-/p53abn tumors (P=0.044). HPV positivity remained an independent prognostic factor for favorable outcome in the multivariable analysis (P=0.020).

Conclusions: HPV- and HPV+ vulvar (pre)cancers display striking differences in somatic mutation patterns. HPV-/p53wt VC appear to be a distinct clinicopathologic subgroup with frequent *NOTCH1* mutations. HPV+ VC have a significantly lower local recurrence rate, independent of clinicopathological variables, opening opportunities for reducing overtreatment in VC.

Introduction

Traditionally, vulvar cancers (VC), of which the majority consist of squamous cell carcinomas, are sub classified depending on the presence or absence of human papilloma virus (HPV). HPV-positive (HPV+) VCs (about 30% of cases) originate in high-grade squamous intra-epithelial lesions (HSIL), formerly referred to as vulvar intraepithelial neoplasia of usual type (uVIN) (1, 2). In Europe, approximately 80% of VCs are HPV negative (HPV-), occur in older women, and are frequently associated with lichen sclerosus (LS) (1, 3). This subtype has been shown to frequently harbour *TP53* mutations. Differentiated vulvar intraepithelial neoplasia (dVIN) has been suggested to be the precancerous lesion preceding this subtype (3). dVIN has a high rate of malignant progression, which is estimated to be as high as 80% (1, 4). Other poorly characterised but putative HPV- VC precursors are verruciform lichen simplex chronicus (VLSC) and vulvar acanthosis with altered differentiation (VAAD) (5).

Few studies have investigated genetic alterations in VCs and its precursor lesions beyond HPV status (6-11). These studies have analysed a limited selection of genes using Sanger sequencing or small panel hotspot approaches (8, 10, 11). Thus far, a more comprehensive assessment of molecular alterations in VC and its precursors is lacking (12). Recent large-scale next generation sequencing (NGS) projects on other tumor types have delivered novel insights with clinical relevance (13-17). For head and neck cancer (HNC), a cancer with many clinico-pathological similarities to VC, large-scale NGS studies (13, 14) have showed that HPV+ and HPV- are molecularly distinct subtypes. Furthermore, these studies have advanced our understanding by identifying novel findings, such as a high frequency of *NOTCH1* mutations in HPV- tumors (13, 14).

The aim of the current study was to characterize the molecular landscape of VCs and their precursor lesions. We have taken advantage of the resemblance between HNC and VC by designing a targeted NGS approach including genes in pathways that have proven to be relevant in HNC. Our NGS results suggest that VCs can be classified into three categories: HPV+, HPV- with a *TP53* mutation and HPV- with wild type *TP53*. To investigate the clinical significance of this categorization, we also examined the clinical behaviour of these groups in a large cohort of 236 patients with VC in whom clinical follow-up was available.

Materials and methods

Sequencing cohort

Tissue samples

VC samples and precursor lesions (dVIN, LS, VAAD and HSIL) were collected from the pathology department in the Leiden University Medical Center. Sample selection was based on original diagnosis described in the pathology report and on the size of the available samples. Non-squamous vulvar cancers were excluded. From eight VC patients, adjacent precursor lesions were available and from one VC patient, sequential biopsies were included in the sample collection. In order to enrich for HPV-independent precursor lesions, an additional nationwide search for the HPV-independent precursor lesion dVIN was performed through the "nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA)" (18). Sample collection was approved by the medical ethics committee of the LUMC (reference number B16.024). All haematoxylin-eosin-stained (H&E) slides were re-evaluated by an expert gynaecologic pathologist (TB) blinded to molecular or immunohistochemical results.

Development of the targeted vulvar NGS panel

A targeted NGS panel (the VC NGS panel) was designed using previously published data on somatic mutations in VC, its precursors (12) and in HNC (13, 14, 19-21). The panel consists of 176 amplicons covering 97% of the coding region of 17 genes with a role in critical cellular pathways, such as differentiation, proliferation and apoptosis. The selected genes are; *BRAF*, *CASP8*, *CDKN2A*, *EZH2*, *FAT1*, *HRAS*, *KMT2C*, *KMT2D, KRAS*, *NOTCH1*, *NOTCH2*, *PIK3CA*, *SYNE1*, *SYNE2*, *NSD1*, *TP53* (covering exon 2-12), *TP63*. The primer sequences were synthesized by Integrated DNA Technology (IDT, Leuven, Belgium) and are available upon request.

DNA extraction

H&E-slides were reviewed by an expert gynaecologic pathologist (TB) who annotated the area, enriching for lesional cells. Unstained 10 μm sections (4 when the tumor was larger than 1 cm and 8 when the tumor was smaller than 1 cm) were cut from FFPE tissue blocks and dried at 37˚C overnight. The sections were deparaffinized in xylol, rehydrated and stained with haematoxylin after which the tumor tissue was manually microdissected based upon the previous annotation on the H&E slide. When possible, associated normal tissue was microdissected separately. After proteinase K digestion overnight, DNA was extracted according to the manufacturers protocol (Nucleospin® DNA FFPE XS, Macherey-Nagel). The obtained DNA was quantified using the Qubit dsDNA broad range assay kit (Life Technologies, Gent, Belgium). A minimum of 50 ng DNA was necessary in order to perform the targeted next generation sequencing (NGS).

Library preparation and sequencing

For library construction 50 ng of DNA was amplified using the primer pool from the designed targeted NGS panel. The samples were barcoded with an adapter and a patient specific barcode in a second round of PCR. After each round of PCR, purification with AmpureXP beads took place. Final sample pooling was based upon the Cq-values acquired with quality PCR. After sample pooling, size selection was performed and final concentration measured with LabOnAChip. Next, emulsion PCR and loading of the chip on the Ion Chef System was done. Subsequently, targeted NGS was performed with the Ion Proton™ System according to the manufacturer's instructions.

Mutation calling

The generated reads were aligned to the human genome (hg19) using the Burrows-Wheeler aligner (BWA, version 0.7.5a) (22). SNP and indel calling was carried out using VarScan software (version v2.3.6) with the following arguments: minimum read depth = 50, minimum number of reads with the alternative allele = 2, minimum base quality = 20, minimum variant allele frequency = 0.10 and p-value <0.01.

Variants were functionally annotated using ANNOVAR (23). We then selected the ones more likely to have a deleterious effect, which was done by focusing on non-sense, frameshift variants and variants known to be of clinical significance or with a cadd_phred score higher 15. Variants with a population frequency higher than 1% in the 1000 Genomes project (24) were removed, since they are more likely to be germline. The called mutations were visually inspected using Integrative Genomics Viewer (IGV) software by LN and DR (http//www.broadinstitute.org.igv).

Follow up cohort

Follow-up data, HPV-status and p53 immunohistochemical data were available from a follow-up cohort consisting of 236 patients with VC. These patients were consecutively treated for primary VC in the LUMC (147 patients) and the Hospital Clinic de Barcelona (89 patients) between 1983 and 2012. A local recurrence was defined as a histologically confirmed recurrence within two years after primary treatment. In the LUMC cohort only recurrences on the ipsilateral side of the vulva were considered a local recurrence.

HPV analysis

DNA extracted from two 10-µm whole tissue sections was used for HPV analysis. To prevent contamination and to serve as a negative control sections of a paraffin block without tissue were cut before each tumor sample. We performed the SPF-10 PCR from the INNO-LiPA HPV Genotyping Extra Amp kit (Innogenetics, Gent, Belgium) according to manufactures protocol to investigate whether or not HPV was present. All blank paraffin sections were negative for HPV in the final PCR analysis. HPV+ cases were

further genotyped using a reverse hybridization line probe assay (LiPA; Innogenetics) through which 25 individual genotypes could be identified. Only samples infected with high-risk HPV were designated as HPV+.

P53 immunohistochemistry

P53 expression in the VC was evaluated by immunohistochemistry . Mutational data for comparison was limited to the sequencing cohort, and not available for the follow-up cohort. Sections of 4 μm thickness were cut from formalin-fixed paraffin-embedded specimen blocks and dried overnight at 37˚C. Tissue sections were stained as described previously (25) using a monoclonal mouse antibody to p53 (Thermo scientific; 1:2000 dilution; clone DO-7). Two pathologists (VS and TB) performed all the scoring and interpretations of the IHC stains. Consensus meetings were held for the samples that were interpreted differently. P53 staining on VC was scored as "wild type" (p53wt) when nuclei of tumor cells stained weak to moderately, comparable to adjacent normal epithelium. Three patterns of staining were defined as "p53 abnormal (p53abn)"; 1) strong overexpression of all tumor cells 2) overexpression in the invasive tumor front or the undifferentiated / non-keratinized basal and parabasal cells at the interface with stroma, regardless of the location of the nests within the tumor mass, 3) completely absent staining in the tumor cells, with positive internal control showing a wild type pattern (26).

Statistical analysis

Statistical analysis was performed with SPSS version 20.0. We divided the patient groups into HPV +, HPV- with a p53 wild type staining pattern (HPV-/p53wt) and HPV- with a p53-abnormal staining pattern (HPV-/p53abn). The chi-square test was used to compare baseline characteristics between groups. Kaplan Meier analysis was performed to estimate local recurrence risk and overall survival. Multivariable analysis was performed with the Cox proportional hazard model and included age, tumor size, depth of invasion and lymph node status.

Results

Sequencing cohort

Sample characteristics

166 samples were collected for genetic characterisation. It was possible to isolate at least 50 ng of DNA for evaluation with targeted NGS from 125 samples. After library preparation and sequencing a total of 119 samples could be analysed (Figure 1). One sample was excluded from the final results due to repeating outlier results. Pathology review (HE only) showed high concordance with local pathology; in 108/118 (92%).

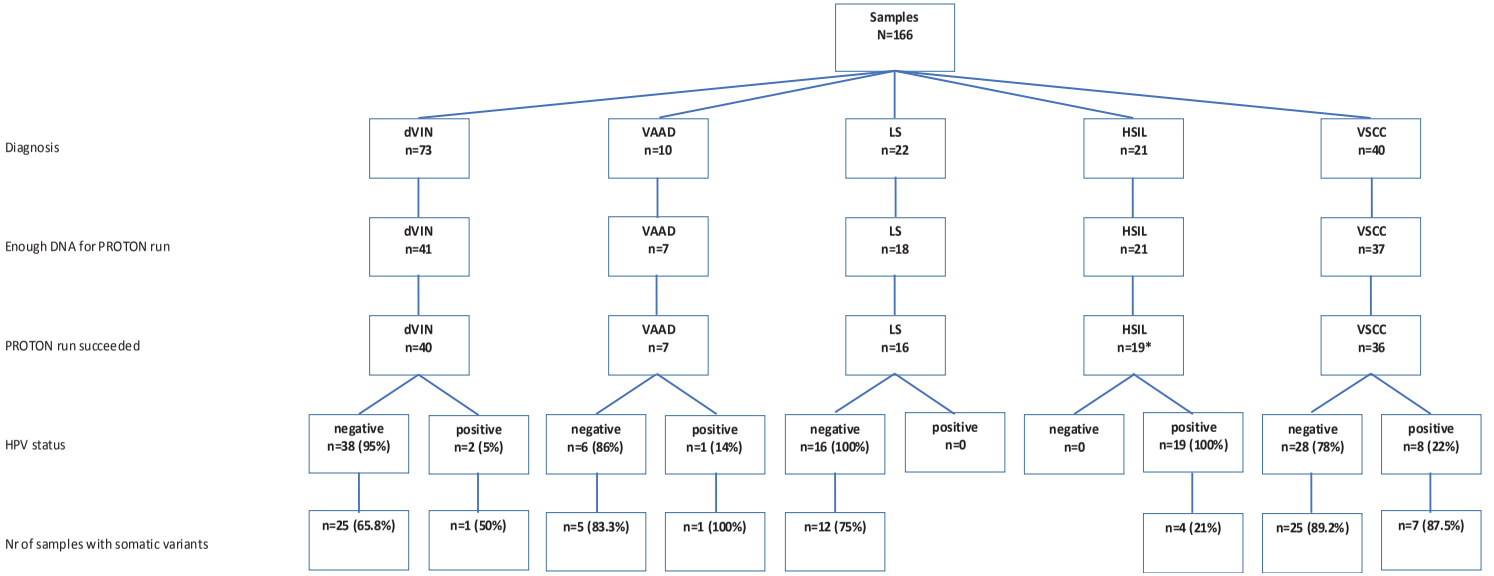


Figure 1: Flowchart sequencing cohort

dVIN: differentiated vulvar intraepithelial neoplasie
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus
HSIL: high grade squamous intraepithelial lesion
VSCC: vulvar squamous cell carcinom
HPV: human papilloma virus
* one sample was excluded from the final analysis due to outlying results

Ten cases were discordant, of which eight dVIN (reclassified to VAAD (5 cases) or LS (3 cases)), one HSIL (reclassified to dVIN) and one LS (reclassified to VAAD). After revision the cohort for sequencing included 118 samples; 40 dVIN, 7 VAAD, 16 LS, 19 HSIL and 36 vulvar cancer samples. The diagnosis after review was used for all further analysis.

Sequence coverage

For the 118 samples analysed, the mean read length of each sequence was 162 bp and the average sequence was 147.7 Mb per sample. There was an average of 5177 reads per amplicon (range 0-496.101). 165/176 (93.8%) amplicons succeeded and 150/176 (85.2%) amplicons had an average of at least 50 reads. *CDKN2A* and *TP63* were removed from the final data analysis due to poor sequence coverage.

Mutational spectrum of precursor lesions and VC in relation to HPV status

The HPV status and somatic mutations found by NGS in relation to the histology are visually represented in a mutational heatmap in figures 2a (precursor lesions) and 2b (VC). Targeted NGS was performed and analysed on 82 precursor lesions; 22/82 (27%) were HPV+ and 60/82 (73%) were HPV-. Somatic mutations were significantly less frequent in HPV+ (7/22, 31.8%) than HPV- precursor lesions (43/60, 71.7%, p-value=0.001) (Figure 2a, table 1 and supplemental table 1). The most commonly mutated gene is *TP53* (26/82, 32% all sequenced precursor lesions; 1/22, 5% HPV+ and 25/60, 42% HPV-), followed by *NOTCH1* (20/82, 24% all sequenced precursor lesions; 3/22, 14% HPV+ and 17/60, 28% HPV-) and *HRAS* (15/82, 18% all sequenced precursor lesions; 3/22, 14% HPV+ and 12/60, 20% HPV-). In the HPV- precursor lesions 35/60 (58%) were *TP53* wild type. A somatic mutation in *NOTCH1* was found in 10/35 (29%) and in *HRAS* in 7/35 (20%) of these HPV-, *TP53* wild type precursor lesions. Most HPV+ cases were histologically classified as HSIL (19/22, 86%). Two of the HPV+ cases were diagnosed as dVIN. These cases retrospectively probably represent HSILs with superimposed inflammatory changes, mimicking dVIN. One HPV+ case was diagnosed as VAAD (2/40, 5% and 1/7, 14%, respectively). None of the LS cases included in this study was HPV+. Somatic mutations were significantly more common in the dVIN (65%), VAAD (86%) and LS (75%) samples, compared to HSIL (25%). Mutations in *TP53* were found in 19/40 (47.5%) dVIN, 1/7 (14.3%) VAAD, 5/16 LS (31.3%) and 2/19 HSIL (10.5%). Mutations in *NOTCH1* and *HRAS* were found in 8/40 (20%) and 4/40 (10%) dVIN, 2/7 (28.6%) and 5/7 (71.4%) VAAD, 8/16 (50%) and 5/16 (31.3%) LS and 2/19 (10.5%) and 1/19 (5.3%) HSIL samples, respectively. Finally, genes with lower mutational frequencies were *MLL2* (7/82, 8.5%), *MLL3* (5/82, 6.1%), *NSD1* (4/82, 4.9%), *NOTCH2* (4/82, 4.9%) and *SYNE1* (5/82, 6.1). Thirty-six VC samples were available for targeted NGS, 7 (19%) were HPV+ and 29 (81%) were

HPV-. Somatic mutations were found in 32/36 (89%) of all vulvar cancer samples (Figure 2b, table 1 and supplemental table 1). The frequency of somatic mutations was the same for HPV+ (6/7, 85.7%) as for HPV- (26/29, 89.7%) VCs. Multiple somatic mutations were less frequent in HPV+ than HPV- VCs (2/7, 28.5% HPV+ and 14/29, 48.2% HPV-). In the total cohort, most mutations were found in *TP53* (21/36, 58.3%). To investigate the potential utility of p53 immunohistochemistry as a surrogate marker for the identification of *TP53* mutational status, we performed p53-IHC on this sequencing cohort (supplemental figure 3). In 31/36 VC the results were concordant, resulting in a kappa of 0.72 (substantial agreement).

Other frequently mutated genes were *NOTCH1* (12/36, 33.3%) and *HRAS* (10/33, 27.8%). Finally, genes with lower mutational frequencies were *CASP8* (3/36, 8.3%), *MLL2* (3/36, 8.3%), *MLL3* (4/36, 11.1%), *NOTCH2* (2/36, 5.6%), *SYNE1* (5/36, 13.9%) and *SYNE2* (2/36, 5.6%). From eight VC patients included in our sequencing cohort adjacent precursor lesions (5 directly adjacent, and 3 distant but in same specimen) could be analysed. The results of these patients are shown in supplemental figure 1. An identical *TP53* mutation was identified in 3/5 precursor lesions directly adjacent to the VC. Interestingly, the somatic mutations in the remaining paired cases were distinctly different from the VC, suggesting that precursor lesions in these cases, despite the close proximity to the VCs, are likely unrelated (supplemental figure 1A).

From one patient interval biopsies and material from a tumor positive lymph node was available. This patient had VC on the right labium in 2001 which was treated surgically (no material available). In 2010 she had a local excision of a lesion from the left side of the vulva that showed a dVIN with possible micro-invasion (sample 1, supplemental figure 1B). In August 2011, she developed a dVIN lesion with possible micro-invasion on the right side of the vulva, which was surgically removed (sample 2). In October 2012, the patient developed VC in the midline of the vulva (sample 3). She underwent local excision combined with a resection of an enlarged left inguinal lymph node (sample 4). The same *NOTCH1* mutation was found in sample 2 and 3. Sample 3 and 4 contained the same mutation in *TP53*. These cases illustrate how the mutational profile may change during tumor progression.

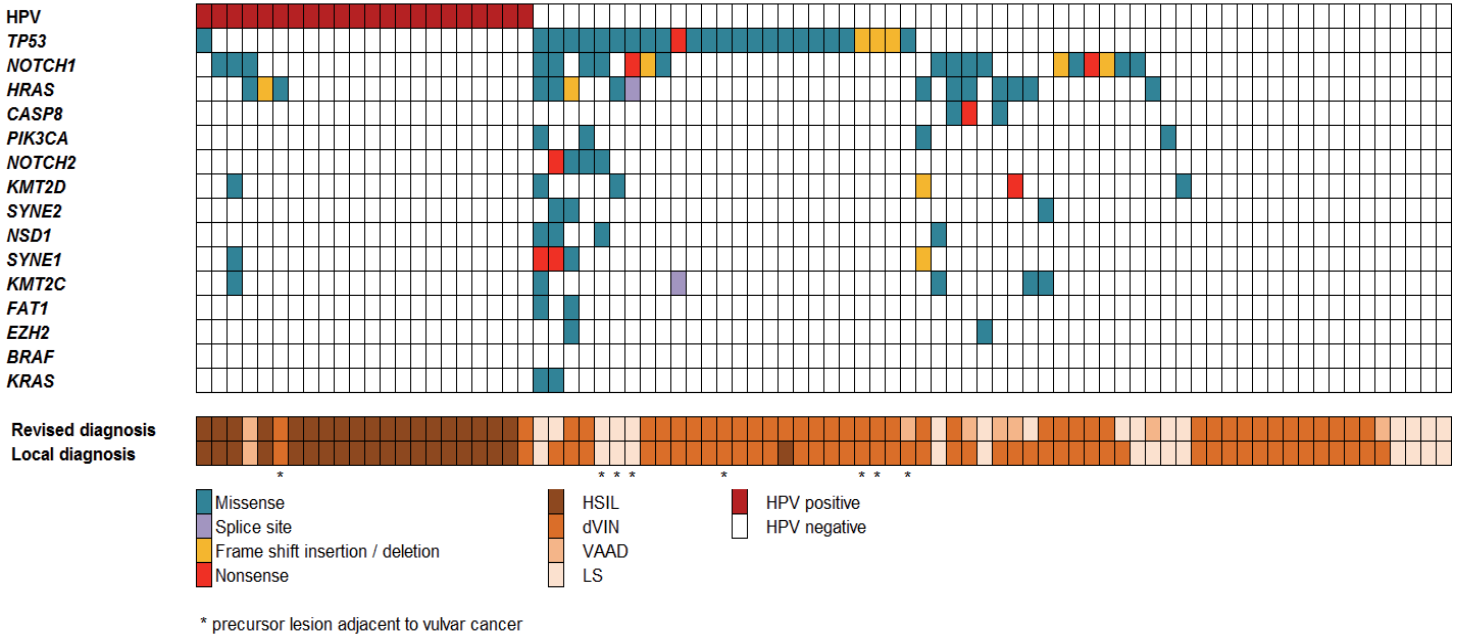


Figure 2a: Mutational spectrum of precursor lesions

HPV: human papillomavirus
HSIL: high grade squamous intraepithelial neoplasia
dVIN: differentiated vulvar intraepithelial neoplasia
LS: lichen sclerosis
*Precursor lesion adjacent to VC

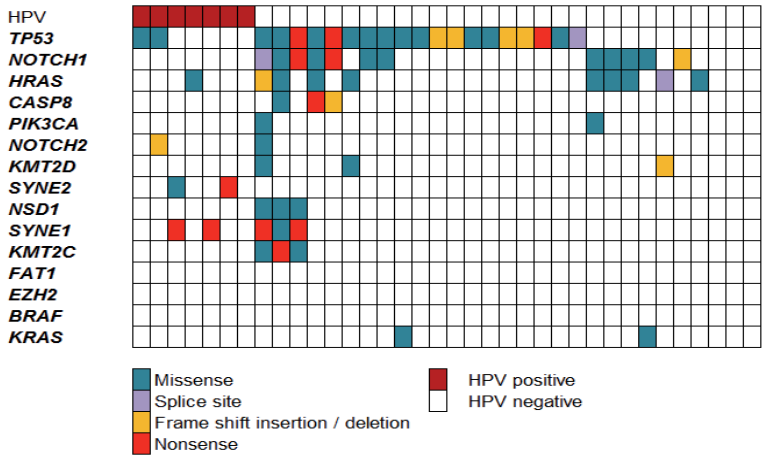


Figure 2b: mutational spectrum of vulvar cancers

Follow-up cohort

Prognostic implication of VC subtypes

Because our NGS results appear to suggest three distinct genetic subtypes of VC, 1) HPV+ VC, 2) HPV-/p53wt VC, and 3) HPV-/p53abn VC, we sought to determine the clinical outcome of this sub classification. For this, we analysed a second cohort of 236 VC patients for the presence of HPV and the expression of p53 by immunohistochemistry (follow-up cohort). Patient characteristics from the follow up cohort are described in table 2. HPV was positive in 38/236 (16.1%) patients and negative in 198/236 (83.9%) patients. In the HPV- group 43/198 (21.7%) had a wild type p53 expression pattern and 155/198 (78.2%) an abnormal p53 expression pattern. Two of the 38 patients with HPV+ tumors (5.3%) developed a local recurrence, whereas 7/43 (16.3%) of the patients with HPV-/p53wt tumors and 35/155 (22.6%) of the patients with HPV-/p53abn tumors developed a local recurrence (Figure 3a, p=0.044). The HPV+ patients were younger, had a lower FIGO stage and less often had tumor positive lymph nodes. When comparing the HPV-/p53wt and HPV-/p53abn groups with each other no clinical or tumor characteristics remained significantly different. There was no difference in local recurrence rate between the HPV-/p53wt and HPV-/p53abn groups (p=0.246). Five year survival was 75% for the patients with HPV+ tumors , 67.2% for the patients with HPV-/p53wt tumors and 56.3% for the patients with HPV-/P53abn tumors (supplemental figure 2, p=0.296). Disease specific survival was better for patients with HPV+ tumors compared to patients with HPV- tumors (Figure 3b, p=0.049). HPV+ status remained an independent favourable prognostic factor in multivariable analysis (Table 3, p=0.020).

Table 1: number of samples with somatic mutations found with the vulvar targeted next generation sequencing panel

	TP53	*BRAF*	*CASP8*	*EZH2*	*FAT1*	*HRAS*	*MLL2 (KMT2D)*	*MLL3 (KMT2C)*	*KRAS*	*NOTCH1*	*NOTCH2*	*NSD1*	*PIK3CA*	*SYNE1*	*SYNE2*
dVIN	19	0	1	1	1	4	2	1	0	8	2	0	2	2	2
(n=40)	47.5%	0%	2.5%	2.5%	2.5%	10%	5%	2.5%	0%	20%	5%	0%	5.0%	5%	5%
VAAD	1	0	2 28.6%	0	0	5	1	0	0	2	0	0	0	0	0
(n=7)	14.3%	0%		0%	0%	71.4%	14.3%	0%	0%	28.6%	0%	0%	0%	0%	0%
LS	5	0	0	1	1	5	3	3	2	8	2	4	2	2	1
(n=16)	31.3%	0%	0%	6.3%	6.3%	31.3%	18.8%	18.8%	12.5%	50%	12.5%	25%	12.5%	12.5%	6.3%
HSIL	1	0	0	0	0	1	1	1	0	2	0	0	0	1	0
(n=19)	5.3%	0%	0%	0%	0%	5.3%	5.3%	5.3%	0%	10.5%	0%	0%	0%	5.3%	0%
VC	21	0	3	0	0	10	3	4	2	12	2	3	2	5	2
(n=36)	58.3%	0%	8.3%	0%	0%	27.8%	8.3%	11.1%	5.6%	33.3%	5.6%	8.3%	5.6%	13.9%	5.6%
HPV-	19	0	3	0	0	9	3	4	2	12	1	3	2	3	0
(n=29)	65.5%	0%	10.3%	0%	0%	31.0%	10.3%	13.8%	6.9%	41.4%	3.4 %	10.3%	6.9%	10.3%	0%
HPV+	2	0	0	0	0	1	0	0	0	0	1	0	0	2	2
(n=7)	28.5%	0%	0%	0%	0%	14.3%	0%	0%	0%	0%	14.3%	0%	0%	28.5%	28.5%
P-value	*0.001*	*NA*	*0.024*	*0.522*	*0.522*	*0.001*	*0.500*	*0.227*	*0.142*	*0.071*	*0.520*	*0.005*	*0.520*	*0.522*	*0.823*

dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosis
HSIL: high grade squamous intraepithelial neoplasia
VC: vulvar cancer
HPV: human papillomavirus
NA: not available

Table 2: Patient characteristics (n=236)

	HPV+ (n=38)	HPV-/p53wt[1] (n=43)	HPV-/p53abn[2] (n=155)	P-value
Age (mean in years)	62 (25-92)	68 (36-93)	74 (37-96)	**<0.001**
FIGO 2009				**0.043**
Stage I	27 (71.1%)	28 (65.1%)	76 (49%)	
Stage II	4 (10.5%)	1 (2.3%)	10 (6.5%)	
Stage III	6 (15.8%)	13 (30.2%)	67 (43.2%)	
Stage IV	1 (2.6%)	1 (2.3%)	2 (1.3%)	
Tumor size				0.083
≤ 40 mm	30 (78.9%)	33 (76.8%)	103 (66.5%)	
> 40mm	5 (13.2%)	9 (20.9%)	47 (30.3%)	
Missing	3 (7.9%)	1 (2.3%)	5 (3.2%)	
Depth of invasion				0.253
≤ 4 mm	17 (44.7%)	17 (39.5%)	49 (31.6%)	
> 4mm	21 (55.3%)	26 (60.5%)	106 (68.4%)	
Primary treatment vulva				**<0.001**
Radical local excision	19 (50%)	26 (60.5%)	42 (27.1%)	
Vulvectomy	19 (50%)	17 (39.5%)	113 (72.9%)	
Tumor positive lymph nodes in the groin(s)	7 (18.4%)	15 (34.9%)	68 (45.6%)	**0.012**
Local recurrence	2 (5.3%)	7 (16.3%)	35 (22.6%)	**0.044**
Current patient status				**0.043**
Alive	26 (68.4%)	26 (60.5%)	84 (54.1%)	
Death	12 (21.6%)	17 (39.5%)	71 (45.9%)	
Median follow up time (months)	57 (2-174)	54 (6-172)	50 (0-206)	0.707

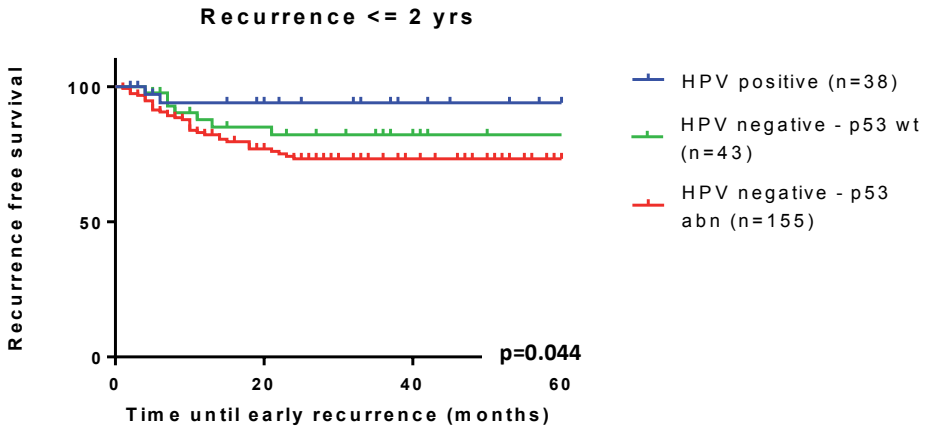


Figure 3a: Kaplan Meyer-curves for local recurrence rate

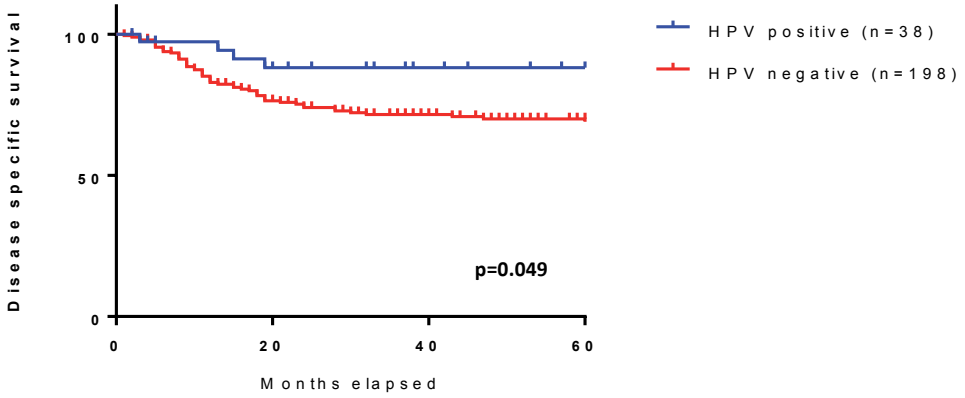


Figure 3b: Kaplan Meyer-curves for disease specific survival based on HPV status

Table 3: Multivariable analysis

Tumor characteristics	Hazard ratio (95% CI)	P-value
Age (mean in years)	1.024 (1.004 – 1.045)	**0.021**
Tumor size		
> 40 mm vs ≤ 40 mm	0.534 (0.291 – 0.981)	**0.043**
Depth of invasion		
> 4 mm vs ≤ 4 mm	2.077 (1.174 – 3.675)	**0.012**
Lymph node status		
Tumor positive yes vs no	1.119 (0.675 – 1.856)	0.663
HPV status		
Positive vs negative	0.287 (0.101 – 0.819)	**0.020**

Discussion

This is the first study using targeted NGS to describe the mutational landscape of vulvar precursor lesions (n=82) and VC (n=36). With this approach, we were able to describe a mutational landscape of vulvar precursor lesions and VC. We found frequent somatic mutations in *TP53, NOTCH1* and *HRAS* in HPV- precursors and VCs. This finding suggests a critical role for these genes in the early development of VC. This is the first report to identify frequent somatic mutations in *NOTCH1* in VC and its precursors. Mutations in *NOTCH1* co-occurred with *TP53* mutations, but were also identified in VCs and precursors that did not carry *TP53* mutations. The frequency of *NOTCH1* and/or *HRAS* mutations was the highest in the HPV- VCs without a *TP53* mutation (7/10, 70%) compared to the HPV- VCs with a *TP53* mutation (8/19, 42%) and HPV+ VCs (1/7, 14%). Similar differences in TP53 mutational rate between HPV- and HPV+ vulvar cancers were recently reported by Weberpals et al (27). In our analysis of the precursor lesions, a strikingly similar pattern was observed suggesting that *NOTCH1* and *HRAS* are likely drivers of vulvar carcinogenesis that can act independently of *TP53*. Therefore, these data support a third molecularly distinct subtype that is HPV independent and *TP53* wild type. In a large follow-up cohort of 236 patients with VC we were able to identify these three subtypes using straightforward, and clinically applicable methods. This approach resulted in significant differences in local recurrence rate in univariable (p=0.044) and multivariable analysis (p=0.020) for patients with HPV+ tumors compared to patients with HPV- tumors.

The finding of somatic mutations in *NOTCH1* (32/118, 27.1%) in VC and precursor lesions is a novel finding of the current study. Interestingly, two recent studies that performed whole-exome and whole-genome sequencing on HNC also identified

frequent *NOTCH1* mutations (13, 14). These studies focused on the genomic differences between HNCs with and without HPV, but did not stratify HPV-, *TP53* wild type from HPV-, *TP53* mutant HNCs. In light of our findings, we analysed these publicly available HNC data for the relation between HPV, *TP53* and *NOTCH1*. We found, similarly to our findings in VCs, that *NOTCH1* mutations in HNC are also predominantly found in HPV-, *TP53* wild type tumors (12/36, 33.3%) compared to HPV-, *TP53* mutant (37/185, 20%) or HPV+ (3/34, 8.8%, data not shown) (13). All these data strongly suggest that aberrant Notch signalling is involved in the carcinogenesis of a subset of HPV- squamous cell carcinomas from both vulvar and head and neck origin. Aberrant notch function has been found in many other tumor types (14, 28-31) and intriguingly is associated with both tumor suppressor as well as an oncogenic function (29, 32). Previous studies found a clear association in HNCs between inactivating mutations in *NOTCH1* and carcinogenesis. This indicates that notch has a primary tumor suppressor function in this tumor type and likely also in VC (14, 21). In line with this, prediction models indicated that most *NOTCH1* mutations identified in our NGS cohort are predicted to be inactivating (data not shown). Furthermore, aberrant notch signalling is proposed to be an early event in mouse models of oesophagus cancer (33). Interesting in this respect are our finding of several lichen sclerosus cases, carrying *NOTCH-1* mutations. Although we didn't have follow-up information on these cases, it is tempting to speculate *NOTCH-1* mutations may predict for progression. Interesting studies on targeted therapies of notch in solid tumors have evolved in the last years, however most often focussed on inhibition of notch signalling. Early-stage clinical trials are investigating inhibition of notch through inhibition of g-secretase (the enzyme responsible for cleavage of notch receptors and downstream signalling) as a potential anticancer therapeutic strategy (34). It will be worth to further investigate the exact role of *NOTCH1* in the carcinogenesis in vulvar cancer, as it might be a novel opportunity for targeted therapy. Another frequently mutated gene worthy of further exploration in VC, was *HRAS*. Previous work already identified somatic mutations in *HRAS* in HPV- VCs and showed an associated with a worse prognosis (6). *HRAS* is an oncogene involved in the RTK/RAS/PI(3)K pathway, and somatic mutations lead to cell proliferation (13, 14).

Previous studies have already noted upon the presence of HPV negative VC that are wild type for *TP53* (9, 10, 35-39). In the current study an in-depth genomic analysis of these VC further support the concept of this third molecular group in VCs. The finding that this third group is also present in HNCs and in vulvar precursor lesions favours this proposed 3-tiered classification. Molecularly this subgroup has the highest frequency of *NOTCH1* and *HRAS* mutations, but also other mutations in other genes were identified. Morphologically most of the HPV-, *TP53* wild type precursors were diagnosed as dVIN and VAAD. A recent report, supportive of our findings, also identified HPV-

and *TP53* wild type vulvar cancers and describe frequent activating *PIK3CA* mutations (73%) in this subset of cancers (40). We identified two *PIK3CA* mutations in our VCs (2/28, 7.1%). To further delineate the molecular characteristics of this HPV-, *TP53* wild type subgroup of VCs more in depth analysis, such as whole exome sequencing, will be required. In the follow-up cohort, the HPV-/p53wt group appeared to have an intermediate risk of recurrence, however this did not reach statistical significance when comparing this to the HPV-/p53abn group (p=0.264, data not shown). A possible explanation is that we were underpowered to detect an effect. Therefore, future larger studies will be required to establish whether the HPV-/p53wt VCs are not only a separate molecular group, but also clinically distinct.

Previous studies on the influence of HPV on prognosis in patients with VC found contradictory results. Some found no difference in local recurrence rate and overall survival (41-43), whereas others were able to find a prognostic benefit for HPV in univariate analysis (44-46). HPV remained a favourable prognosticator in multivariable analysis in only two other studies (45, 46). Although our study does not fulfil all criteria for a biomarker study (REMARK criteria) (47), it is the largest series of VC patients to date and shows the prognostic benefit of HPV in multivariable analysis. This finding is supported by a recently published study by McAlpine et al, who found a superior progression free survival and disease specific survival for patients with HPV+ VC in a cohort treated after 1995. Taken together the results of these studies, we can now put this discussion to rest and can conclude the HPV+ VC have a significant better clinical behaviour. Next, we need to discuss whether these finding should have consequences for the treatment of patients with VC. Interestingly, McAlpine et al noted that HPV status in a cohort treated before 1995, did not show a difference in outcome. This may suggest that the more conservative surgical approach that has been developed in the course of the years has led to worse outcomes for patients with HPV- VC (48). Currently, all patients with VC, irrespective of HPV status, are treated similarly, with surgery being the first choice of treatment (49). The outcome of the present study raises the question whether HPV testing (or p16 IHC, as an excellent surrogate (48)) should be performed on all VC biopsies to identify patients with HPV- tumors with a high risk of recurrence. A possible clinical implication might be to perform a more radical surgical procedure when HPV is not detected followed by a more stringent follow-up scheme due to the higher chance of developing a local recurrence. Furthermore, HPV status might also be utilized as a predictive marker for response to adjuvant treatment (48, 50). For HNC, where the favourable prognosis of HPV+ cancers also has been established, (51, 52) prospective studies are ongoing to investigate whether adjuvant chemotherapy can be omitted in HPV+ tumors (53, 54).

Of course, also this study has its limitations, our targeted NGS design relies on the parallels between VC and HNC. This leads to a directed search for somatic mutations but of course limits the discovery of novel gene mutations. Additionally, we were dealing with small biopsies of vulvar precursor lesions, limiting the extend of the DNA analysis. Unfortunately, we had to remove *CDKN2A* from our panel due to poor sequencing coverage. We were therefore unable to report on the frequency of *CDKN2A* mutations. Finally, we were not able to associate our molecular findings of the precursor lesions to clinical follow-up. Future studies should be designed to determine the possible prognostic capacity of somatic mutations in the progression to VCs.

In conclusion, this report is the first to establish a genetic landscape of a large cohort of VCs and precursors using targeted NGS. We identified a distinct mutational profile in HPV+ VCs and give a molecular description of a group of HPV-independent VCs without *TP53* mutations. This third molecular subtype of VC shows a high frequency of *NOTCH1* and *HRAS* mutations and appear to have its own precancerous lesions, morphologically in the spectrum of dVIN and VAAD. Using a large cohort of patients with VC with long term follow-up, we were able to identify HPV as a significant favourable prognostic factor. P53 status seems to further refine local recurrence risk in the HPV-independent VCs. The recognition that VCs can be classified in at least three distinct molecular subgroups using clinically applicable markers represents a promise for risk stratification and opens opportunities for precision medicine for patients with vulvar cancer.

References

1. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. Crit RevOncolHematol. 2008;68(2):131-56.
2. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. Histopathology. 2013;62(1):161-75.
3. van der Avoort IA, Shirango H, Hoevenaars BM, Grefte JM, de Hullu JA, de Wilde PC, et al. Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways. IntJGynecolPathol. 2006;25(1):22-9.
4. McAlpine JN, Kim SY, Akbari A, Eshragh S, Reuschenbach M, von Knebel Doeberitz M, et al. HPV-independent Differentiated Vulvar Intraepithelial Neoplasia (dVIN) is Associated With an Aggressive Clinical Course. Int J Gynecol Pathol. 2017.
5. Nascimento AF, Granter SR, Cviko A, Yuan L, Hecht JL, Crum CP. Vulvar acanthosis with altered differentiation: a precursor to verrucous carcinoma? AmJSurgPathol. 2004;28(5):638-43.
6. Trietsch MD, Spaans VM, ter Haar NT, Osse EM, Peters AA, Gaarenstroom KN, et al. CDKN2A(p16) and HRAS are frequently mutated in vulvar squamous cell carcinoma. GynecolOncol. 2014;135(1):149-55.
7. Rolfe KJ, MacLean AB, Crow JC, Benjamin E, Reid WM, Perrett CW. TP53 mutations in vulval lichen sclerosus adjacent to squamous cell carcinoma of the vulva. BrJCancer. 2003;89(12):2249-53.
8. Soufir N, Queille S, Liboutet M, Thibaudeau O, Bachelier F, Delestaing G, et al. Inactivation of the CDKN2A and the p53 tumour suppressor genes in external genital carcinomas and their precursors. BrJDermatol. 2007;156(3):448-53.
9. Pinto AP, Miron A, Yassin Y, Monte N, Woo TY, Mehra KK, et al. Differentiated vulvar intraepithelial neoplasia contains Tp53 mutations and is genetically linked to vulvar squamous cell carcinoma. ModPathol. 2010;23(3):404-12.
10. Choschzick M, Hantaredja W, Tennstedt P, Gieseking F, Wolber L, Simon R. Role of TP53 mutations in vulvar carcinomas. Int J Gynecol Pathol. 2011;30(5):497-504.
11. Holway AH, Rieger-Christ KM, Miner WR, Cain JW, Dugan JM, Pezza JA, et al. Somatic mutation of PTEN in vulvar cancer. ClinCancer Res. 2000;6(8):3228-35.
12. Trietsch MD, Nooij LS, Gaarenstroom KN, van Poelgeest MI. Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: a review of the current literature. GynecolOncol. 2015;136(1):143-57.
13. Cancer Genome Atlas Research N. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-82.
14. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157-60.
15. Cancer Genome Atlas Research N, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. Nature. 2013;497(7447):67-73.
16. McDaniel AS, Stall JN, Hovelson DH, Cani AK, Liu CJ, Tomlins SA, et al. Next-Generation Sequencing of Tubal Intraepithelial Carcinomas. JAMA Oncol. 2015;1(8):1128-32.

17. Dallol A, Buhmeida A, Al-Ahwal MS, Al-Maghrabi J, Bajouh O, Al-Khayyat S, et al. Clinical significance of frequent somatic mutations detected by high-throughput targeted sequencing in archived colorectal cancer samples. J Transl Med. 2016;14(1):118.
18. Casparie M, Tiebosch AT, Burger G, Blauwgeers H, van de Pol A, van Krieken JH, et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. Cellular oncology : the official journal of the International Society for Cellular Oncology. 2007;29(1):19-24.
19. Gaykalova DA, Mambo E, Choudhary A, Houghton J, Buddavarapu K, Sanford T, et al. Novel insight into mutational landscape of head and neck squamous cell carcinoma. PLoS One. 2014;9(3):e93102.
20. Pickering CR, Zhang J, Yoo SY, Bengtsson L, Moorthy S, Neskey DM, et al. Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. Cancer Discov. 2013;3(7):770-81.
21. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154-7.
22. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 2012;22(3):568-76.
23. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38(16):e164.
24. Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, et al. A global reference for human genetic variation. Nature. 2015;526(7571):68-74.
25. Nooij LS, Dreef EJ, Smit VT, van Poelgeest MI, Bosse T. Stathmin is a highly sensitive and specific biomarker for vulvar high-grade squamous intraepithelial lesions. J Clin Pathol. 2016;69(12):1070-5.
26. McCluggage WG, Soslow RA, Gilks CB. Patterns of p53 immunoreactivity in endometrial carcinomas: 'all or nothing' staining is of importance. Histopathology. 2011;59(4):786-8.
27. Weberpals JI, Lo B, Duciaume MM, Spaans JN, Clancy AA, Dimitroulakos J, et al. Vulvar Squamous Cell Carcinoma (VSCC) as Two Diseases: HPV Status Identifies Distinct Mutational Profiles Including Oncogenic Fibroblast Growth Factor Receptor 3. Clin Cancer Res. 2017.
28. Maliekal TT, Bajaj J, Giri V, Subramanyam D, Krishna S. The role of Notch signaling in human cervical cancer: implications for solid tumors. Oncogene. 2008;27(38):5110-4.
29. Rettig EM, Chung CH, Bishop JA, Howard JD, Sharma R, Li RJ, et al. Cleaved NOTCH1 Expression Pattern in Head and Neck Squamous Cell Carcinoma Is Associated with NOTCH1 Mutation, HPV Status, and High-Risk Features. Cancer PrevRes(Phila). 2015;8(4):287-95.
30. Agrawal N, Jiao Y, Bettegowda C, Hutfless SM, Wang Y, David S, et al. Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. Cancer Discov. 2012;2(10):899-905.
31. Sakamoto K, Fujii T, Kawachi H, Miki Y, Omura K, Morita K, et al. Reduction of NOTCH1 expression pertains to maturation abnormalities of keratinocytes in squamous neoplasms. Lab Invest. 2012;92(5):688-702.

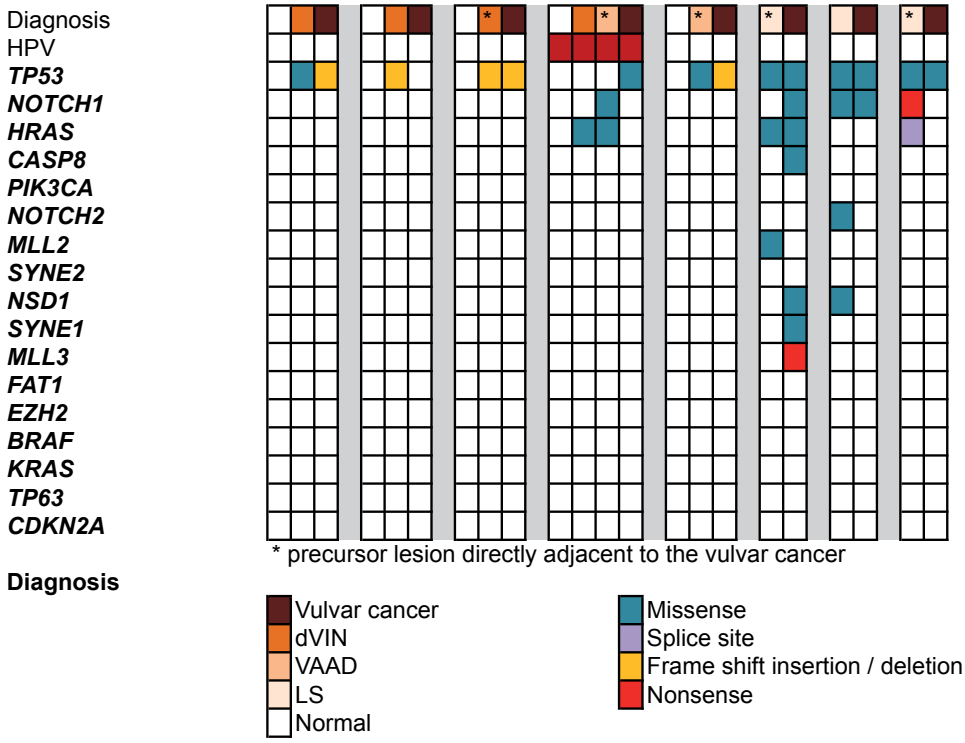
32. Yap LF, Lee D, Khairuddin A, Pairan MF, Puspita B, Siar CH, et al. The opposing roles of NOTCH signalling in head and neck cancer: a mini review. Oral Dis. 2015;21(7):850-7.
33. Nowell CS, Radtke F. Notch as a tumour suppressor. Nat Rev Cancer. 2017;17(3):145-59.
34. Weaver AN, Burch MB, Cooper TS, Della Manna DL, Wei S, Ojesina AI, et al. Notch Signaling Activation Is Associated with Patient Mortality and Increased FGF1-Mediated Invasion in Squamous Cell Carcinoma of the Oral Cavity. Mol Cancer Res. 2016;14(9):883-91.
35. Lee YY, Wilczynski SP, Chumakov A, Chih D, Koeffler HP. Carcinoma of the vulva: HPV and p53 mutations. Oncogene. 1994;9(6):1655-9.
36. Pilotti S, D'Amato L, Della TG, Donghi R, Longoni A, Giarola M, et al. Papillomavirus, p53 alteration, and primary carcinoma of the vulva. Diagn MolPathol. 1995;4(4):239-48.
37. Kim YT, Thomas NF, Kessis TD, Wilkinson EJ, Hedrick L, Cho KR. p53 mutations and clonality in vulvar carcinomas and squamous hyperplasias: evidence suggesting that squamous hyperplasias do not serve as direct precursors of human papillomavirus-negative vulvar carcinomas. HumPathol. 1996;27(4):389-95.
38. Flowers LC, Wistuba II, Scurry J, Muller CY, Ashfaq R, Miller DS, et al. Genetic changes during the multistage pathogenesis of human papillomavirus positive and negative vulvar carcinomas. J Soc Gynecol Investig. 1999;6(4):213-21.
39. Kashofer K, Regauer S. Analysis of full coding sequence of the TP53 gene in invasive vulvar cancers: Implications for therapy. Gynecol Oncol. 2017.
40. Watkins JC, Howitt BE, Horowitz NS, Ritterhouse LL, Dong F, MacConaill LE, et al. Differentiated exophytic vulvar intraepithelial lesions are genetically distinct from keratinizing squamous cell carcinomas and contain mutations in PIK3CA. Mod Pathol. 2016.
41. Alonso I, Fuste V, Del PM, Castillo P, Torne A, Fuste P, et al. Does human papillomavirus infection imply a different prognosis in vulvar squamous cell carcinoma? GynecolOncol. 2011;122(3):509-14.
42. Pinto AP, Schlecht NF, Pintos J, Kaiano J, Franco EL, Crum CP, et al. Prognostic significance of lymph node variables and human papillomavirus DNA in invasive vulvar carcinoma. Gynecol Oncol. 2004;92(3):856-65.
43. van de Nieuwenhof HP, van Kempen LC, de Hullu JA, Bekkers RL, Bulten J, Melchers WJ, et al. The etiologic role of HPV in vulvar squamous cell carcinoma fine tuned. Cancer Epidemiol Biomarkers Prev. 2009;18(7):2061-7.
44. Hay CM, Lachance JA, Lucas FL, Smith KA, Jones MA. Biomarkers p16, Human Papillomavirus and p53 Predict Recurrence and Survival in Early Stage Squamous Cell Carcinoma of the Vulva. J Low Genit Tract Dis. 2016;20(3):252-6.
45. Lindell G, Nasman A, Jonsson C, Ehrsson RJ, Jacobsson H, Danielsson KG, et al. Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node. GynecolOncol. 2010;117(2):312-6.
46. Monk BJ, Burger RA, Lin F, Parham G, Vasilev SA, Wilczynski SP. Prognostic significance of human papillomavirus DNA in vulvar carcinoma. ObstetGynecol. 1995;85(5 Pt 1):709-15.
47. Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. J Natl Cancer Inst. 2009;101(21):1446-52.

48. McAlpine JN, Leung S, Cheng A, Miller D, Talhouk A, Gilks CB, et al. HPV-independent vulvar squamous cell carcinoma has a worse prognosis than HPV-associated disease: a retrospective cohort study. Histopathology. 2017.
49. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.
50. Lee LJ, Howitt B, Catalano P, Tanaka C, Murphy R, Cimbak N, et al. Prognostic importance of human papillomavirus (HPV) and p16 positivity in squamous cell carcinoma of the vulva treated with radiotherapy. Gynecol Oncol. 2016;142(2):293-8.
51. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. IntJCancer. 2007;121(8):1813-20.
52. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. JNatlCancer Inst. 2008;100(4):261-9.
53. Su W, Liu J, Miles BA, Genden EM, Misiukiewicz KJ, Posner M, et al. Adjuvant Radiation Therapy Alone for HPV Related Oropharyngeal Cancers with High Risk Features. PLoS One. 2016;11(12):e0168061.
54. Dietz A, Wichmann G, Wiegand S. Should We De-escalate the Treatment for HPV-Positive Tumors? Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 2017;206:173-81.

Supplementary data

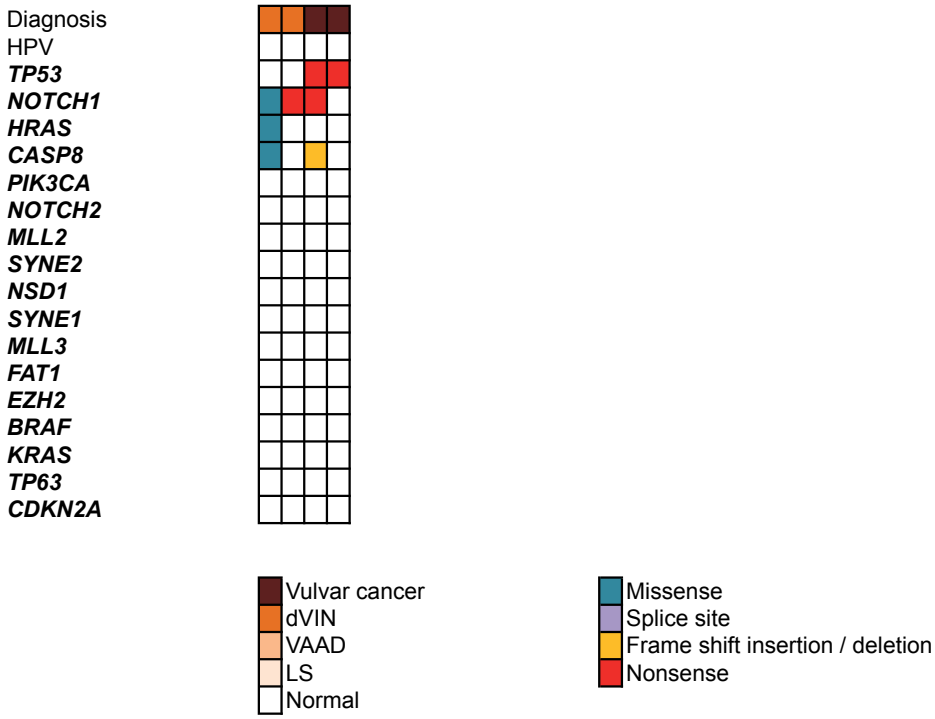
Supplemental table 1

Available upon request



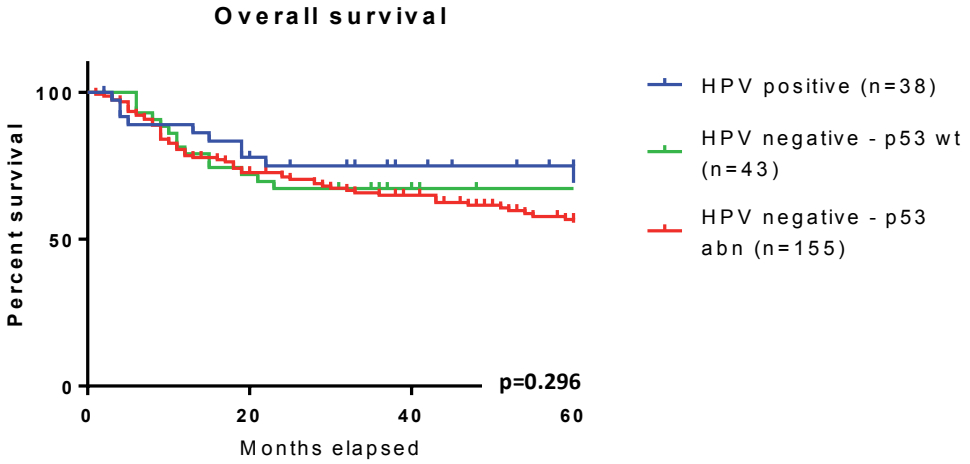
Supplemental figure 1a: Overview of somatic mutations found in normal and precursor lesion, adjacent to vulvar cancer

HPV: human papillomavirus
dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus

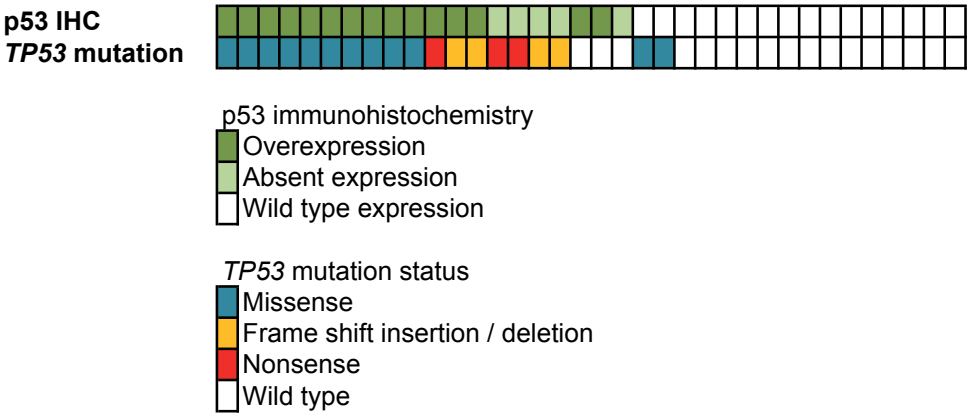


Supplemental figure 1b: Follow-up of one patient over time

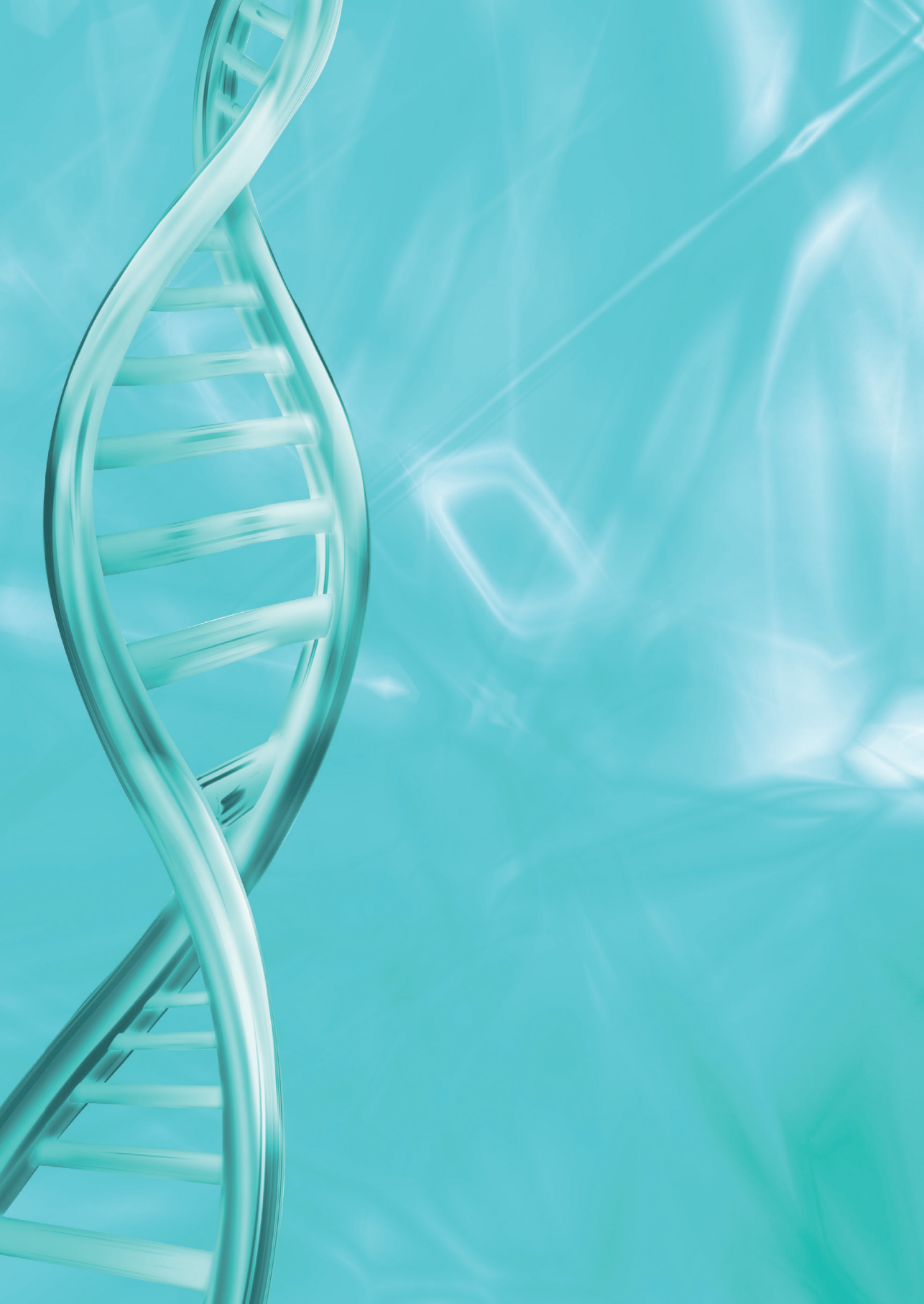
HPV: human papillomavirus
dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus



Supplemental figure 2: overall survival. HPV: human papillomavirus



Supplemental figure 3: overview of concordance between p53 immunohistochemistry and *TP53* mutation status in 36 vulvar cancer samples included in the sequencing cohort. Kappa = 0.72



CHAPTER 8

General discussion and summary

General discussion and summary

Despite major advances in the past decade, treatment of vulvar cancer (VC) remains challenging and is still associated with significant mortality and morbidity. VC is a cancer type with a particularly high age of onset, with a peak incidence around seventy years of age. The fact that this cancer predominantly affects older women has important implications for treatment and recovery, as co-morbidities are not infrequent. VC is also a rare cancer with only around 300 new cases a year in the Netherlands. Due to this rarity, this cancer subtype is under researched and little is known about the carcinogenesis and its molecular features compared to other more frequently occurring cancers.

For this thesis we intended to shed light on some significant clinical issues as well as advancing our basic understanding of VC. The work presented in this thesis follows the current trend in medical oncology, in which we have sought for avenues towards individualising treatment for this particularly fragile patient population. In the first section, we challenge current guidelines regarding the extent of the surgical procedure for both the primary lesions as well as the groin area. Also, treatment options in case of a recurrence are discussed. In the second section the discussion continues with studies that increase our understanding of the VC carcinogenesis (e.g. initiating events and driver alterations) and how this may provide avenues towards personalised treatment.

Section I. Clinical challenges in the treatment of vulvar cancer (chapter 2, 3 and 4)
In the first section of this thesis we have focussed on two critical questions regarding the primary surgery of VC. In chapter 2 we asked ourselves what the limits are for safe tumour-free margins in VC. Arguably, this is the most important question in primary surgery of VC, because there's a fine balance between being radical and overtreatment. In recent years, following a trend seen in many other tumour types, surgery of VC has become more and more conservative (1, 2). The question of the minimal tumour-free margin has been asked before. At this moment a tumour-free margin of ≥ 8 mm is considered the norm to prevent local recurrence, which has been adopted in many guidelines (3-8). In our investigation we challenged this advice and examined whether a tumour-free margin of < 8 mm is indeed associated with an increased chance of developing a local recurrence as compared to ≥ 8 mm (***chapter 2***). In this study we first performed a meta-analysis of current available literature and found a clear increase in local recurrence risk in the group of patients with a tumour-free margin of < 8 mm (pooled risk ratio 1,99, p=0.02), supporting the current guidelines (3, 4, 9, 10). However, the studies included in this meta-analysis were heterogeneous regarding tumour and treatment characteristics. A particular weakness we noticed was the lack of a clear definition of local recurrence. We then decided to perform a cohort study on VC patients treated in the LUMC, using a strict definition for local recurrence. We

defined a local recurrence as a histologically confirmed recurrence of VC within 2 years, located on the ipsilateral side of the vulva. In this cohort study we found that the chance of a (strictly defined) local recurrence was not different in patients with a tumour-free margin of < 8 mm (10%) versus ≥ 8 mm (12%). In fact, patients truly at high risk of a (strictly defined) local recurrence are those with tumour-positive margins (31%). From this we concluded that aiming for a tumour-free margin of 8 mm might be too stringent. This was further supported by a recent large study (11) on 289 VC patients with FIGO stage IB and higher, in which the authors found a local recurrence rate of 12.6% for patients with a tumour-free margin of < 8 mm and 10.2% for patients with a tumour-free margin of ≥ 8 mm (p=0.392). None of the patients received adjuvant treatment after primary treatment (11).

Despite these convincing results on the limited role of a minimal tumour-free margin of 8 mm for prevention of local recurrence, clinicians are reserved in changing current guidelines and adjusting treatment strategy. The data presented in **chapter 2** and the study of Woelber et al. (11) argue that a tumour-free margin of < 8 mm should not be the determining factor for adjuvant treatment. Other tumour characteristics, such as tumour size, the presence of lymph vascular space invasion and tumour-positive lymph nodes have proven to be much stronger prognosticators with regard to the development of a local recurrence, and therefore these factors should determine the decision for re-excision or adjuvant radiotherapy (12-14). However, for the field to make such a change, the data on the prognostic impact of the tumour-free margins are probably insufficient. Not only are the available data conflicting, they are also based on retrospective cohorts. To overcome these limitations, a possible next step could be to perform a prospective randomised controlled trial aiming to investigate the benefit of adjuvant treatment for patients with a tumour-free margin of < 8 mm. Such a trial can be named 'Surgical Margins in the Treatment of Vulvar Cancer' (the SuMaToV-trial, figure 1). All patients who are surgically treated for primary VC can be included in the trial. Patients without an indication for adjuvant treatment based upon clinical or tumour characteristics other than a tumour-free margin of <8mm will be randomised between adjuvant treatment (standard arm) or no adjuvant treatment (experimental arm). Adjuvant treatment in the standard arm should consist of re-excision when possible or otherwise radiotherapy in accordance with the current guidelines (5-8). After a minimum of two year follow-up the first results can be analysed. The primary outcome is recurrence free survival. Secondary outcomes are treatment related morbidity and overall survival. Local recurrences should be registered according to a previous established strict definition as we used in our study; histologically confirmed recurrence on the ipsilateral side of the vulva within two years after primary treatment. As a translational component to this study, molecular analysis to define clonal relationship with the primary tumour may be considered. Treatment

related morbidity and overall survival should be registered in all patient groups. Through such a prospective study a final answer on this important clinical question is possible.

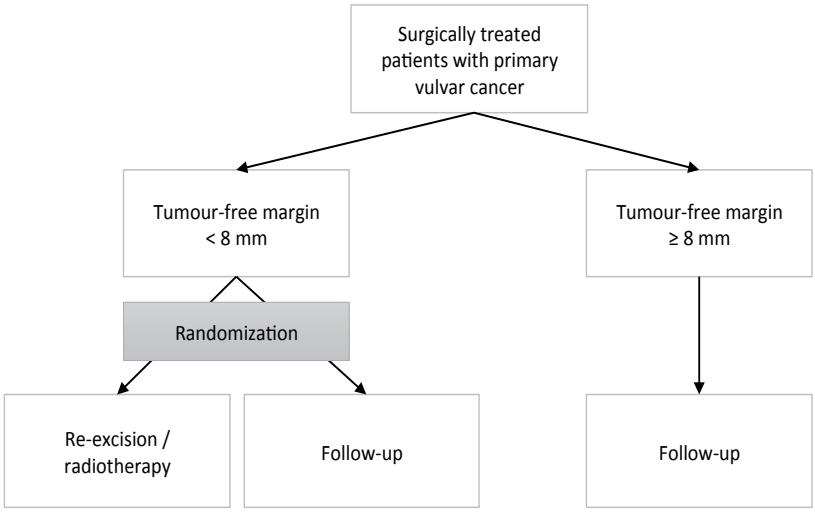


Figure 1: Proposed radomization strategy for the SuMaToV trial (Surgical Margins in the Treatment of Vulvar cancer)

Of course, our strict definition for local recurrence can be debated. So far, there is no golden standard definition, and therefore our definition is based upon common sense and the experience that most recurrences develop within two years after primary treatment (40-80%) (15, 16). Interestingly, a recently published long term follow-up study from the GROningen INternational Study on Sentinel nodes in Vulvar cancer (GROINNS-V) also found a relatively high percentage of late recurrences. Median time until local recurrence was 27 months and local recurrence rate was 27.2% 5 years after primary treatment and even 39.5% 10 years after primary treatment. Most of these local recurrences occurred more than two years after primary treatment (63.9%) (17). It can be argued, however, that many of these late recurrences are second primary tumours instead of true recurrences. It is assumed that the complete vulva is at risk for the development of multiple tumours due to a so called "field effect" or "field cancerization". This assumption is supported by the clinical course of VC patients. It is not uncommon that VC patients present with multifocal tumours on the vulva, which are probably unrelated to each other. The concept of field cancerization is not unique to the vulva, and has also been described in other organ systems where (pre-)neoplastic processes are present at multiple sites. For example, field cancerization is a concept used in several other organ systems such as head and neck, lung, esophagus, cervix, colon, breast, bladder and skin (18-23). It has been shown that a contiguous (epi)genetically altered field can be the basis of multiple genetically related but independent lesions,

which probably should be regarded as second primary tumours rather than local recurrences. Such a field has been shown for metachronous lesions that were > 7 cm apart (18, 22). Given the strong association between VC and chronic lichen sclerosus (LS), LS may be regarded as "the field" that predisposes for the development of latent vulvar precancerous lesions. Whether these latent precancerous lesions progress is likely dependent on the acquisition of additional genetic alterations, which in turn result in subclones with uncontrolled cellular proliferation, such as *TP53* mutations in dVIN. Eventually, these subclones are likely to evolve into invasive cancer (18, 24). This sequence of events can occur at multiple different sites within the fields and at different points in time. Although data in support of this model in VC are still limited, it appears very likely to be applicable in this disease too.

The presence of a field has important implications when we consider the above described studies on local recurrences. We have proposed a clinical definition of true recurrence (ipsilateral and within 2 years), reflecting those lesions that are the result of incomplete removal of the primary tumour. New lesions that occur at the contralateral side of the vulva or after more than 2 years are unlikely the result of inadequate primary surgery, but rather the consequence of an incompletely excised field at risk. We currently don't know how to recognise, demarcate and remove or treat this field to prevent second primary tumours to occur, which would be a topic of great interest for future research. Precancerous lesions such as differentiated vulvar intraepithelial neoplasia (dVIN) or vulvar acanthosis with altered differentiation (VAAD) in the margins are currently not an indication for re-excision. In our study on the value of the histological margin (***chapter 2***) we also evaluated the influence of dVIN presence in the resection margin on local recurrence rate. We were not able to prove that the presence of dVIN increases local recurrence risk. Still, given the above described hypothesis, this seems plausible and should be further investigated in a more comprehensive study. In a study on 28 patients with head and neck squamous cell carcinoma (HNSCC) all margins of the surgical specimen were analysed to determine the extension of a genetically altered field. Genetic alterations were detected in 10/28 (36%) of the patients and in 7 patients these alterations were present in the surgical margins. After a median follow-up time of twelve months, none of these patients had developed a local recurrence (22).

Field cancerization might also explain the differences found in our meta-analysis (4, 9-11, 25, 26) and cohort study (***chapter 2***). The width of the tumour-free margin does not influence the chance of developing a true recurrence, a histologically confirmed recurrence within two years after primary treatment and on the ipsilateral side of the vulva. On the other hand, a tumour-free margin of > 8 mm increases the chance of removing "the field" and thereby theoretically decreases the chance of developing second primary tumours. This might explain the results found in our cohort study, in which

we held on to a very strict definition of local recurrence. The studies included in our meta-analysis often did not define a local recurrence, which probably means that all new tumours on the vulva were seen as local recurrences, independent of time or localization on the vulva.

Contradictory to our proposal for less radical surgery in the SuMaToV trial, but supportive for the presence of a field is a theory proposed by Höckel et al (27). The authors have studied early embryology to analyse local tumour spread and found that the pattern of local tumour spread for cervical and VC is confined by compartments defined by their embryonic development. This is called ontogenetic anatomy and the compartment theory (27, 28). Crossing the border of these compartments is a relative late step during malignant progression. In order to do this phenotypical changes of the tumour cells are necessary. Following this theory even more radical surgery to maintain local tumour control would be required. In one of their studies the authors performed vulvar field resection based upon the ontogenetic anatomy in 54 VC patients in order to investigate if this surgical approach results in an improvement of local tumour control. After a median follow-up time of nineteen months, none of the patients had developed a local recurrence. Unexpectedly, perioperative complication rate was low (29). So, perhaps indeed even more extensive surgery is necessary for prevention of a local recurrence as well as second primary tumours. On the other hand, less radical surgery will increase the chance of a second primary tumour, but reduces morbidity. The question arises whether the morbidity associated with more extensive surgery outweighs the benefits of preventing recurrent disease and thus which approach is best for the patient and results in a better overall survival.

In order to further investigate this it is necessary to make a genuine differentiation between true local recurrences and second primary tumours based upon molecular features rather than an arbitrary clinical definition. The development of our VC Next Generation Sequencing (NGS) panel (***chapter 7***) may serve this purpose as it can provide objective molecular data that can be used to define clonal relationships between two lesions, although a correct distinction between true recurrences and second primary tumours will be challenging and perhaps impossible in some cases. Hypothetically, a true local recurrence most likely will have an identical mutational profile, potentially accompanied with one or two additional somatic mutations. This would be the most likely situation based upon the hypothesis that a true local recurrence develops from tumour cells that were left behind during primary surgery. Second primary tumours will show a different mutational profile compared to the mutational profile of the previous tumour. These second primary tumours have developed after a different second hit elsewhere in the vulnerable field. If we are able to make a genuine distinction between true recurrences and second primary tumours we might also be able to implement this

difference in clinical practise and to advise different treatment strategies. Literature on HNSCC has shown that a second primary tumour has a more aggressive course than true local recurrences (23). This may also be true for VC patients which suggests that more radical surgery is indicated for patients with a second primary tumour followed by more stringent follow-up in comparison to patients with a true local recurrence.

A second clinical challenge in which the extent of surgery is under debate is the primary treatment of the groins in patients with VC, as extensive groin surgery is associated with high morbidity (30, 31). Yet, adequate treatment of the groins is critical, because a recurrence in the groin(s) is associated with an exceptional high mortality rate of up to 90% (32, 33). This high mortality rate is confirmed in our study on groin surgery in VC patients, in which we describe a nine times increased chance of dying for patients who develop a groin recurrence compared to patients who did not develop a groin recurrence (***chapter 3***). The introduction of the sentinel node (SN) procedure as a treatment alternative for the groins has proven to be safe and led to a dramatic decrease in postoperative morbidity of groin treatment (31, 34). Still, approximately half of the patients do not fulfil the criteria for undergoing a SN-procedure, i.e. a unifocal tumour, smaller than 4 cm (31, 34). For patients with a multifocal tumour and/or a tumour larger than 4 cm more extensive treatment is necessary. Currently, most guidelines advise a full inguinofemoral lymfadenectomy (IFL) for all these patients (2, 31). However, our analysis of the risk of recurrence in lymph node positive VC patients shows that nodal debulking followed by radiotherapy is a safe alternative treatment for patients with clinically suspicious lymph nodes and/or macrometastases (***chapter 3***). Our findings are supported by a previous study published by Hyde et al (35), with the difference that our study also addressed the morbidity in these patients. Our study shows a reduction in short term and long term postoperative morbidity in patients treated with nodal debulking, without adversely influencing the chance of developing a recurrence in the groin(s). A recently published review thoroughly investigated different surgical approaches and postoperative morbidity in VC patients who underwent an IFL. The authors found an overall post-operative wound complication rate of up to 85%. Furthermore, the authors found that this complication rate can be reduced slightly following specific surgical techniques such as: using separate incisions, unilateral IFL, sparing of the saphenous vein, preservation of the fascia lata and continuous skin sutures (31). Still, based on our study and the study from Hyde et al. (35) we propose nodal debulking to be the preferred treatment in patients with clinically suspicious lymph nodes and/or macrometastases, resulting in lower morbidity than full IFL. This advice is not yet included in the current guidelines (5, 6, 8). When the guidelines are revised these studies should be included in composing an advise.

The third clinical challenge in the treatment of VC addressed in this thesis, is the optimal treatment when VC does recur. The chance of developing a recurrence is high (12-37%) (12, 15) and this is relevant, as 5-year survival dramatically decreases for patients who develop a recurrence (25-50% versus 50-90% for patients with primary VC). The prognosis of patients with recurrent VC has not improved over the years (8, 13, 36). Therefore, there is an ongoing discussion on how to treat recurrent disease. In order to structure this discussion, this thesis provides an overview of up-to-date literature on the treatment of recurrent VC in order to give an evidence based advise for treatment of recurrent VC (***chapter 4***). In the context of a local recurrence, there is general consensus, that when feasible re-resection with clear margins is the treatment of choice. If surgery is not an option, (chemo)radiotherapy is a good alternative. Patients with a local recurrence with a depth of infiltration > 1mm are advised a full IFL when primary treatment of their VC did not comprise a full IFL (15, 37). This treatment strategy of the groins causes high morbidity rates and the question arises whether the SN-procedure is also a good alternative when treating patients with a local recurrence. Alternative treatment strategies are currently being investigated. A recently published study found that a repeat SN-procedure is feasible, although technically challenging (38). The GROINSS-V study group is aiming to investigate the safety of the SN-procedure for patients with a local recurrence in the next national GROINSS-V trial. The outcomes of this trial will probably contribute in further reducing treatment related morbidity if it proves that this procedure is also safe for patients with a local recurrence (34). In this context it might also be clinically relevant to distinguish between true local recurrences and second primary tumours.

Treatment of a groin recurrence is even more challenging, especially because a groin recurrence used to be considered as almost always fatal (14, 15). Yet, a recent study found a 50% survival rate for patients with a groin recurrence after 7 years and concludes that treatment of a groin recurrence is no longer merely palliative (39). At this moment, the advised treatment for a groin recurrence is surgery, consisting of either a full IFL or debulking, followed by radiotherapy when possible (33). Due to the improved survival rates for patients with a groin recurrence (39) further developments in the treatment of a groin recurrence are highly important.

Pathogenesis of VC (chapter 5, 6 and 7)

The second section of this thesis concentrates on the pathogenesis of VC with a focus on genetic alterations that might be involved. A gynaecologist in the outward patient clinic can encounter patients with various forms of vulvar complaints in different stages of vulvar disease. Patients who present with a vulvar precursor lesion are at risk of developing a VC in the course of their lives. The chance of developing VC depends on the type of the precursor lesion. Knowledge of the underlying mechanisms of initiation

and progression from a precursor lesion towards an invasive lesion is limited, and may inform preventive strategies. Up until now, VCs have been subdivided into two different biological subtypes; those that are associated with high risk Human Papilloma Virus (hrHPV) and those that are not (40-42).This dichotomy view, however, may be too simplistic and particularly little is known about the initiating and early driving events in the pathogenesis of non-HPV associated cancers

For HPV-dependent VC, in many ways the literature parallels that of HPV-dependent cervical cancer and head and neck cancer (43, 44). In that respect, the current application of HPV vaccination in the prevention and treatment of cervical cancer may also be used to prevent and treat VC (44). The nomenclature for vulvar precursor lesions has been somewhat confusing, however currently the hrHPV precursor vulvar high-grade squamous intraepithelial lesions (HSIL) is the preferred term (formerly known as usual VIN 2/3). Differentiation between HSIL, which has a 9-16% chance of progression to VC when left untreated (45) and low-grade squamous intraepithelial lesions (LSIL) (46, 47) is important, since vulvar LSILs are not pre-cancerous and treatment is only necessary if a patient has complaints (48). In ***chapter 6*** we investigated the diagnostic value of stathmin immunohistochemistry (IHC) as an adjunct marker to differentiate between LSIL and HSIL, and found a high sensitivity and specificity for HSIL lesions. Therefore, stathmin expression can be used as an additional marker in difficult cases, in which p16 and Ki67 are not conclusive (49).

Little is known about the pathogenesis of HPV-independent VCs and their precursor lesions called "differentiated VIN (dVIN)" (42, 45). Recent work, clearly demonstrates that dVIN has a high malignant potential, with 80% of all dVINs reported to progress to (invasive) cancer (42, 45, 50). Given the high malignant potential of untreated dVIN it is important to recognise these lesions early and assure patients of adequate treatment and follow-up. At this moment immunohistochemical staining with p53 is commonly used as a marker for dVIN, because *TP53* is frequently mutated in dVIN lesions. In other cancers, an aberrant expression pattern of p53 (either complete absent staining or a strong diffuse staining pattern) has been shown to be an excellent surrogate marker for *TP53* mutation (51). P53 IHC is therefore in pathology practice often used to differentiate between (HPV-associated) vulvar HSIL and dVIN (52). An interesting question that was raised during our studies was whether p53-IHC would also be a surrogate marker for the presence of a *TP53* mutation in the context of VCs. In this light we investigated the p53 staining pattern in the 36 VC patients in our NGS cohort (***chapter 7***) and compared these results with the mutational *TP53* status. Although this is a limited cohort size, we found a substantial concordance (kappa = 0.72) between IHC and NGS. Extension of these data is probably possible for vulvar precursor lesions and therefore, p53-IHC may be an easy to implement surrogate marker for *TP53* mutations in vulvar precursor lesions. We did

notice that p53 staining patterns in *TP53* mutant vulvar cancers can vary, and educating gyneacopathologists will be required, as the interpretation of p53 staining is somewhat different from ovarian or endometrial adenocarcinomas. Recognizing the specific p53-IHC patterns will require further study and will likely improve the kappa, and thereby the utility of p53-IHC as a an adequate surrogate.

Chapter 5 of this thesis gives an overview of the current literature on (epi)genetic alterations and summarizes available molecular data in vulvar (pre)cancer thus far. Clearly, the (epi)genetic landscape of VC, and particularly its major precursor dVIN is largely unknown and limited to some studies confirming frequent *TP53* mutations (53-55). Therefore, we next aimed to explore the mutational landscape of vulvar (pre)cancer using targeted NGS (***chapter 7***). We found a high mutation frequency in HPV-independent dVIN and LS lesions in *TP53* (48% and 31%, respectively), *NOTCH1* (20% and 50%, respectively) and *HRAS* (10% and 31%, respectively). Interestingly, *HRAS* and *NOTCH1* mutations were relatively frequent in vulvar precancers that were *TP53* wildtype. The recurrent *NOTCH1* mutations in VC was a novel finding in this study. The exact role of *NOTCH1* in vulvar (pre)cancers remains uncertain. Reports on *NOTCH1* function describe *NOTCH1* as an oncogene as well as a tumour suppressor gene, depending on the tissue type. The canonical Notch pathway is probably oncogenic and mainly involved in cell proliferation, differentiation and survival (56). Dysregulated Notch plays a crucial role in tumour development by altering the developmental state of a cell and consequently maintaining the cells in a proliferative or undifferentiated state (57).

The findings in the HPV-independent VCs largely overlapped with the findings in the precancerous lesions with recurrent somatic mutations in *TP53*, *NOTCH1* and *HRAS*. This suggests that these gene alterations are likely relevant early events in the development of VC and supports a, not previously appreciated, third molecular subtype of VC. This subtype is HPV-independent and does not carry a pathogenic *TP53* mutation. In our study 10 of 29 VCs (35%) were HPV-independent and *TP53* wildtype. Earlier studies had implicitly identified this subtype, but did not give it any attention (53, 58, 59). Interestingly, HPV-independent and *TP53* wildtype cancers have also been identified in two large studies on HNSCC (60, 61), a tumour type that greatly resembles the oncogenesis of vulvar cancers. In the TCGA-study on 279 HNSCC , 36 cancers (13%) fell within the category (60). The earlier published study by Stransky et al. on 74 HNSCC patients, identified 16 (22%) of these cancers (61). A recent study in which full coding sequencing of *TP53* was performed found no somatic mutation in *TP53* in 14/59 (24%) HPV-independent VCs, supporting a third VC subtype (58). The initiating events and genetic alterations driving this subtype are unknown, but our work supports a role for *NOTCH1* and *HRAS* mutations in this subtype. Our study was limited to targeted mutational data, and therefore lacks information on genes that were

not in our panel. Therefore, we may have missed relevant copy number alterations or epigenetic changes, which should be a theme of future studies.

So, following the results from ***chapter 7***, we can speculate on a refined VC oncogenesis model (Figure 2) in which we also incorporated field cancerization. We propose that LS is the oncogenic field of vulvar epithelial surface required to initiate tumorigenesis. This chronic inflammation results in an increased burden on the basal epithelial keratinocytes, effected the fidelity of DNA replication. This results in areas in which (epi)genetic changes accumulate and result in latent precancerous without a specific histological substrate. Subsequently, when the basal keratinocytes in these latent precancers encounter a somatic mutation in *TP53* or in *NOTCH1* the carcinogenesis is accelerated and results in histologically changes that fall within the spectrum of dVIN/VAAD. In the absence of *TP53* mutation it is possible that additional genomic alterations are required to progress towards invasive VC, however a pathogenic *TP53* mutations is likely sufficient for invasion. This model would favour resection of not only the invasive cancer, but also any visible precancer, in order to reduce the chance of a true recurrence. Second primary tumours arising from the oncogenic field and its latent precancers can't be prevented unless the field is completely excised .

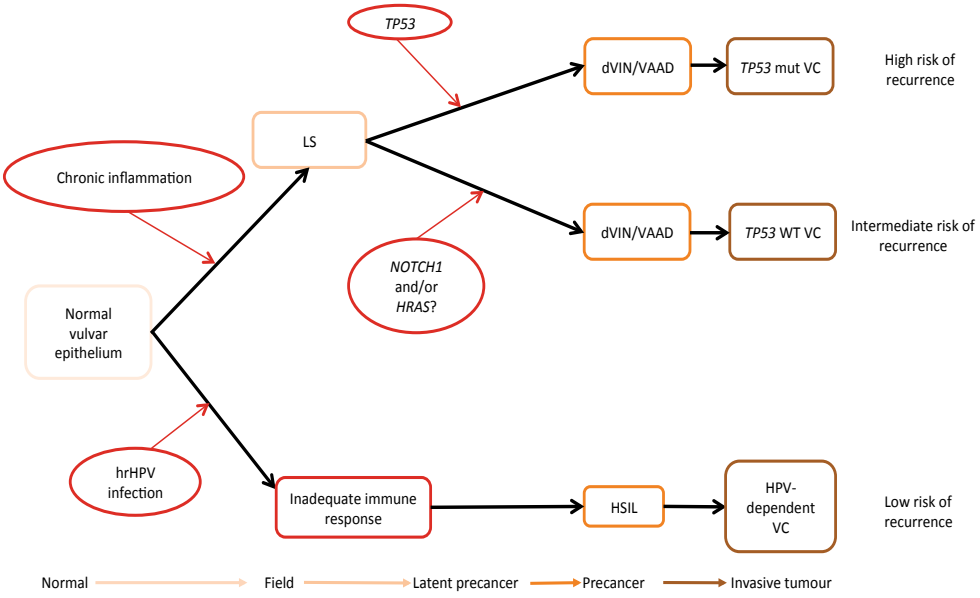


Figure 2: Proposed model for the pathogenesis of vulvar cancer

LS: lichen sclerosus, dVIN: differentiated vulvar intraepithelial neoplasia, VAAD: vulvar acanthosis with altered differentiation, mut: mutant, WT: wildtype, VC: vulvar cancer, hrHPV: high risk human papilloma virus, HSIL: high grade squamous intraepithelial lesion

Obviously, a model of three molecular subtypes would only be of clinical value, if these three subtypes display a differential clinical behaviour (eg. risk of recurrence and or differential treatment response). Therefore, we evaluated the prognostic value of hrHPV on local recurrence rate and overall survival in **chapter 7**. We found a significant improved prognosis for HPV-dependent VCs compared to HPV-independent VCs. The group of patients with HPV-dependent VC developed a local recurrence in 5.3% of the patients and had a better disease specific five-year survival (p-value 0.049). HPV remained a favourable prognostic factor in multivariable analysis (hazard ratio 0.29, p-value 0.02), despite the association with better clinico-pathological characteristics. These results are supported by a recently published study by McAlpine et al, who also found a better progression free and disease specific survival in 217 patients with HPV-dependent VC (62). Previous studies on tumours that greatly resemble the pathogenesis of VC, i.e. penile squamous cell carcinoma (PSCC) and HNSCC also show comparable results, with an unequivocal difference in prognosis between HPV-dependent and HPV-independent patient groups. Patients with HPV-dependent tumours have less recurrences and a better overall survival (63, 64). Given these results it is tempting to consider universal HPV testing for patients with VC. However, due to the retrospective nature of these studies, the question whether the indolent behavior is independent of treatment remains unresolved. Currently, all patients with VC, irrespective of HPV status, are treated identical. Interestingly, studies in HNSCC patients have shown a better response of HPV-dependent tumours on adjuvant treatment (chemotherapy and radiotherapy) (64-66). In addition, one recently published study on 57 VC patients treated with radiotherapy with or without surgical resection found a better progression free and overall survival for patients with HPV-dependent tumours, suggesting sensitivity to radiation (67). Although further research in a prospective cohort is necessary to validate these outcomes, these results are promising and may inform future trial designs. It appears that patients with HPV-dependent VC may benefit from less extensive primary surgery and are more likely to respond to radiation. This may become relevant for both local treatment as well as for treatment of the groins. Furthermore, follow-up schemes of patients with HPV-dependent VC might be less intensive because of the minimal chance of developing a recurrence. In addition to a prognostic benefit for HPV-dependent VC patients, we evaluated the influence of aberrant or normal p53 staining on prognosis in the HPV-independent VC patients. Local recurrence rate was 16.3% for HPV-independent tumours with a p53 normal staining pattern and 22.6% for HPV-independent tumours with an aberrant staining pattern for p53. This difference was not significant (p-value 0.246), probably due to the number of patients included in our cohort. Expansion of a VC cohort with adequate follow-up will provide further insights on the clinical value of distinguishing three VC subtypes in future research.

Conclusion

In conclusion, the molecular heterogeneity of VCs offers novel avenues for the introduction of more individualized treatment strategies in the near future. HPV status and the presence of *TP53* mutations will likely become critical variables required to determine the extent of primary treatment and the necessity of adjuvant treatment, as well as the treatment strategy for recurrent VC. Furthermore, targeted therapy against certain somatic mutations as well as immune therapy will probably undergo a huge development in the next decades and will undoubtedly become part of the treatment plan of VC patients. These developments can contribute to a better prognosis for these patients and to less invalidating surgical and adjuvant treatment. To get these novel developments to our patients, however, prospective trials in which molecular analyses are an integral part, will be required.

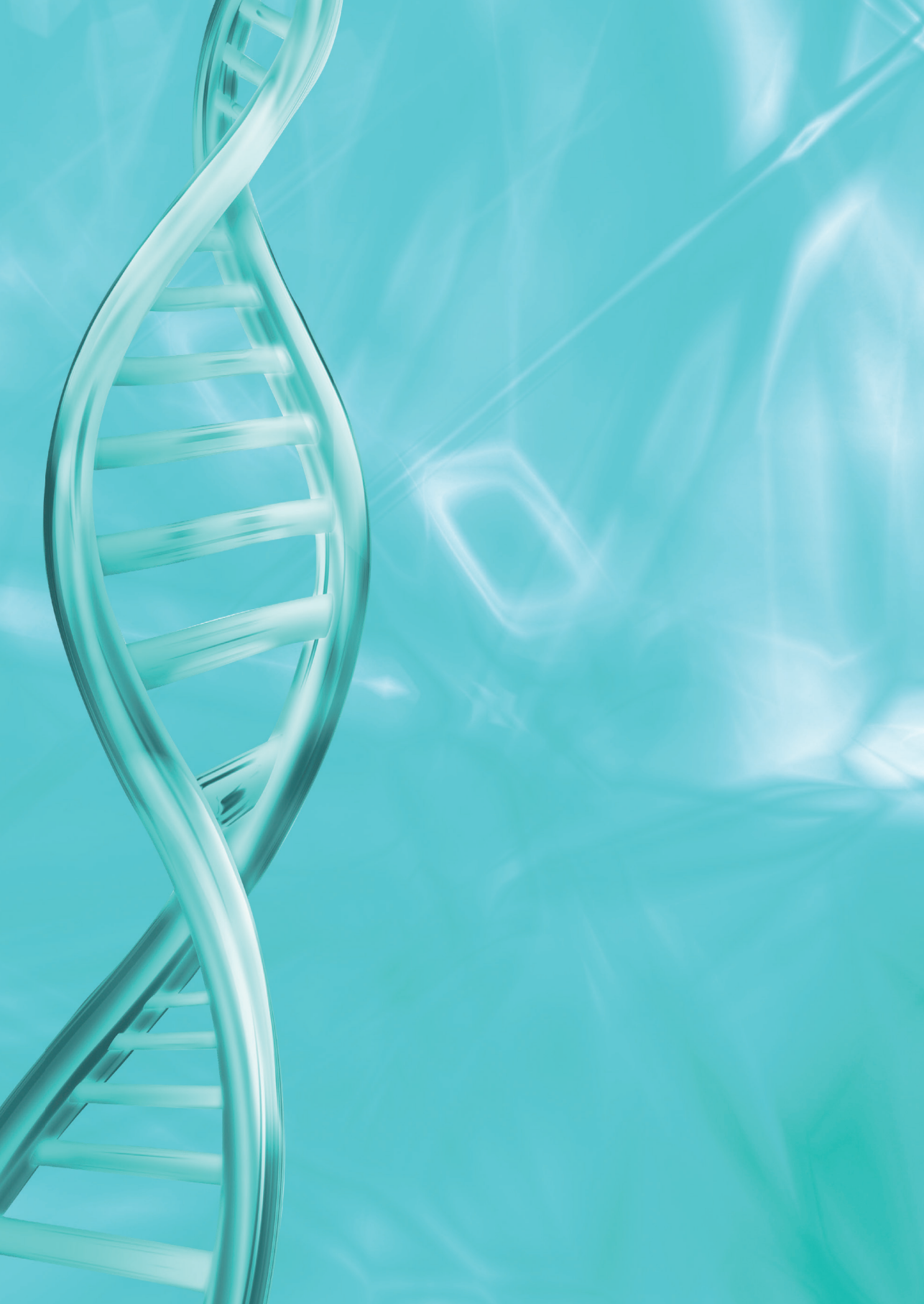
References

1. Hacker NF. Current management of early vulvar cancer. AnnAcadMedSingapore. 1998;27(5):688-92.
2. Hacker NF, Eifel PJ, van d, V. Cancer of the vulva. IntJGynaecolObstet. 2012;119 Suppl 2:S90-S6.
3. Heaps JM, Fu YS, Montz FJ, Hacker NF, Berek JS. Surgical-pathologic variables predictive of local recurrence in squamous cell carcinoma of the vulva. Gynecol Oncol. 1990;38(3):309-14.
4. de Hullu JA, Hollema H, Lolkema S, Boezen M, Boonstra H, Burger MP, et al. Vulvar carcinoma. The price of less radical surgery. Cancer. 2002;95(11):2331-8.
5. National Dutch Guideline Gynaecologic Tumors; Vulvar Carcinoma. http://www.oncoline.nl ed2015.
6. RCOG guideline vulval carcinoma. https://www.rcog.org.uk/globalassets/documents/guidelines/vulvalcancerguideline.pdf ed2015.
7. National Comprehensive Cancer Network guideline on vulvar cancer. https://www.nccn.org/store/login/login.aspx?ReturnURL=http://www.nccn.org/professionals/physician_gls/pdf/vulvar.pdf ed2016.
8. Guideline vulvar cancer, national cancer institute. 2015.
9. Chan JK, Sugiyama V, Pham H, Gu M, Rutgers J, Osann K, et al. Margin distance and other clinico-pathologic prognostic factors in vulvar carcinoma: a multivariate analysis. Gynecol Oncol. 2007;104(3):636-41.
10. Groenen SM, Timmers PJ, Burger CW. Recurrence rate in vulvar carcinoma in relation to pathological margin distance. Int J Gynecol Cancer. 2010;20(5):869-73.
11. Woelber L, Griebel LF, Eulenburg C, Sehouli J, Jueckstock J, Hilpert F, et al. Role of tumour-free margin distance for loco-regional control in vulvar cancer-a subset analysis of the Arbeitsgemeinschaft Gynakologische Onkologie CaRE-1 multicenter study. Eur J Cancer. 2016;69:180-8.
12. Gadducci A, Tana R, Barsotti C, Guerrieri ME, Genazzani AR. Clinico-pathological and biological prognostic variables in squamous cell carcinoma of the vulva. Crit RevOncolHematol. 2012;83(1):71-83.
13. Preti M, Ronco G, Ghiringhello B, Micheletti L. Recurrent squamous cell carcinoma of the vulva: clinicopathologic determinants identifying low risk patients. Cancer. 2000;88(8):1869-76.
14. Woelber L, Mahner S, Voelker K, Eulenburg CZ, Gieseking F, Choschzick M, et al. Clinicopathological prognostic factors and patterns of recurrence in vulvar cancer. Anticancer Res. 2009;29(2):545-52.
15. Coulter J, Gleeson N. Local and regional recurrence of vulval cancer: management dilemmas. Best Pract Res Clin Obstet Gynaecol. 2003;17(4):663-81.
16. Gadducci A, Cionini L, Romanini A, Fanucchi A, Genazzani AR. Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer. Crit RevOncolHematol. 2006;60(3):227-41.
17. Te Grootenhuis NC, van der Zee AG, van Doorn HC, van d, V, Vergote I, Zanagnolo V, et al. Sentinel nodes in vulvar cancer: Long-term follow-up of the GROningen INternational Study on Sentinel nodes in Vulvar cancer (GROINSS-V) I. GynecolOncol. 2016;140(1):8-14.
18. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. Cancer Res. 2003;63(8):1727-30.

19. Torezan LA, Festa-Neto C. Cutaneous field cancerization: clinical, histopathological and therapeutic aspects. AnBrasDermatol. 2013;88(5):775-86.
20. Dakubo GD, Jakupciak JP, Birch-Machin MA, Parr RL. Clinical implications and utility of field cancerization. Cancer Cell International. 2007;7 :15 Mar 2007.
21. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6(5):963-8.
22. Tabor MP, Brakenhoff RH, van Houten VM, Kummer JA, Snel MH, Snijders PJ, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res. 2001;7(6):1523-32.
23. Califano J, Westra WH, Meininger G, Corio R, Koch WM, Sidransky D. Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. Clin Cancer Res. 2000;6(2):347-52.
24. Eva LJ, Ganesan R, Chan KK, Honest H, Malik S, Luesley DM. Vulval squamous cell carcinoma occurring on a background of differentiated vulval intraepithelial neoplasia is more likely to recur: a review of 154 cases. J Reprod Med. 2008;53(6):397-401.
25. Baiocchi G, Mantoan H, de Brot L, Badiglian-Filho L, Kumagai LY, Faloppa CC, et al. How important is the pathological margin distance in vulvar cancer? Eur J Surg Oncol. 2015;41(12):1653-8.
26. Catanzarite T, Neubauer N, Doll K, Singh D, Schink J, Lurain J. Margin status as predictor of recurrence in squamous cell carcinoma of the vulva: Challenging the 8 millimeter rule. International Journal of Gynecological Cancer. 2014;Conference: 14th Biennial Meeting of the International Gynecologic Cancer Society:October 2012.
27. Hockel M, Horn LC, Illig R, Dornhofer N, Fritsch H. Ontogenetic anatomy of the distal vagina: relevance for local tumor spread and implications for cancer surgery. Gynecol Oncol. 2011;122(2):313-8.
28. Hockel M, Horn LC. The puzzle of close surgical margins is not puzzling. Gynecologic Oncology. 2013;130(1):224-5.
29. Hockel M, Schmidt K, Bornmann K, Horn LC, Dornhofer N. Vulvar field resection: novel approach to the surgical treatment of vulvar cancer based on ontogenetic anatomy. Gynecol Oncol. 2010;119(1):106-13.
30. Gaarenstroom KN, Kenter GG, Trimbos JB, Agous I, Amant F, Peters AA, et al. Postoperative complications after vulvectomy and inguinofemoral lymphadenectomy using separate groin incisions. IntJGynecolCancer. 2003;13(4):522-7.
31. Pouwer AW, Arts HJ, van der Velden J, de Hullu JA. Limiting the morbidity of inguinofemoral lymphadenectomy in vulvar cancer patients; a review. Expert Rev Anticancer Ther. 2017:1-10.
32. Oonk MH, Hollema H, de Hullu JA, van der Zee AG. Prediction of lymph node metastases in vulvar cancer: a review. IntJGynecolCancer. 2006;16(3):963-71.
33. Cormio G, Loizzi V, Carriero C, Cazzolla A, Putignano G, Selvaggi L. Groin recurrence in carcinoma of the vulva: management and outcome. EurJCancer Care (Engl). 2010;19(3):302-7.
34. van der Zee AG, Oonk MH, de Hullu JA, Ansink AC, Vergote I, Verheijen RH, et al. Sentinel node dissection is safe in the treatment of early-stage vulvar cancer. JClinOncol. 2008;26(6):884-9.

35. Hyde SE, Valmadre S, Hacker NF, Schilthuis MS, Grant PT, van d, V. Squamous cell carcinoma of the vulva with bulky positive groin nodes-nodal debulking versus full groin dissection prior to radiation therapy. IntJGynecolCancer. 2007;17(1):154-8.
36. Faul C, Miramow D, Gerszten K, Huang C, Edwards R. Isolated local recurrence in carcinoma of the vulva: Prognosis and implications for treatment. International Journal of Gynecological Cancer. 1998;8(5):409-14.
37. Salom EM, Penalver M. Recurrent vulvar cancer. Curr TreatOptions Oncol. 2002;3(2):143-53.
38. Doorn HC, van Beekhuizen HJ, Gaarenstroom KN, van d, V, van der Zee AG, Oonk M, et al. Repeat sentinel lymph node procedure in patients with recurrent vulvar squamous cell carcinoma is feasible. GynecolOncol. 2016.
39. Frey JN, Hampl M, Mueller MD, Gunthert AR. Should Groin Recurrence Still Be Considered as a Palliative Situation in Vulvar Cancer Patients?: A Brief Report. Int J Gynecol Cancer. 2016;26(3):575-9.
40. Del Pino M, Rodriguez-Carunchio L, Ordi J. Pathways of vulvar intraepithelial neoplasia and squamous cell carcinoma. Histopathology. 2013;62(1):161-75.
41. van der Avoort IA, Shirango H, Hoevenaars BM, Grefte JM, de Hullu JA, de Wilde PC, et al. Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways. IntJGynecolPathol. 2006;25(1):22-9.
42. Preti M, Scurry J, Marchitelli CE, Micheletti L. Vulvar intraepithelial neoplasia. BestPractResClinObstetGynaecol. 2014;28(7):1051-62.
43. Sano D, Oridate N. The molecular mechanism of human papillomavirus-induced carcinogenesis in head and neck squamous cell carcinoma. Int J Clin Oncol. 2016;21(5):819-26.
44. Crosbie EJ, Einstein MH, Franceschi S, Kitchener HC. Human papillomavirus and cervical cancer. Lancet. 2013;382(9895):889-99.
45. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. Crit RevOncolHematol. 2008;68(2):131-56.
46. Bornstein J. The 2015 Internation Society for the Study of Vulvovaginal Disease Terminology of Vulvar Squamous Intraepithelial Lesions. 2015.
47. RJ K, ML C, CS H, RH Y. WHO classification of Tumours of Female Reproductive Organs. Lyon: IARC; 2014 2014.
48. Nelson EL, Bogliatto F, Stockdale CK. Vulvar Intraepithelial Neoplasia (VIN) and Condylomata. ClinObstetGynecol. 2015;58(3):512-25.
49. McCluggage WG. Premalignant lesions of the lower female genital tract: cervix, vagina and vulva. Pathology. 2013;45(3):214-28.
50. McAlpine JN, Kim SY, Akbari A, Eshragh S, Reuschenbach M, von Knebel Doeberitz M, et al. HPV-independent Differentiated Vulvar Intraepithelial Neoplasia (dVIN) is Associated With an Aggressive Clinical Course. Int J Gynecol Pathol. 2017.
51. Kobel M, Piskorz AM, Lee S, Lui S, LePage C, Marass F, et al. Optimized p53 immunohistochemistry is an accurate predictor of TP53 mutation in ovarian carcinoma. J Pathol Clin Res. 2016;2(4):247-58.

52. Singh N, Leen SL, Han G, Faruqi A, Kokka F, Rosenthal A, et al. Expanding the morphologic spectrum of differentiated VIN (dVIN) through detailed mapping of cases with p53 loss. AmJSurgPathol. 2015;39(1):52-60.
53. Flowers LC, Wistuba II, Scurry J, Muller CY, Ashfaq R, Miller DS, et al. Genetic changes during the multistage pathogenesis of human papillomavirus positive and negative vulvar carcinomas. J Soc Gynecol Investig. 1999;6(4):213-21.
54. Gasco M, Sullivan A, Repellin C, Brooks L, Farrell PJ, Tidy JA, et al. Coincident inactivation of 14-3-3sigma and p16INK4a is an early event in vulval squamous neoplasia. Oncogene. 2002;21(12):1876-81.
55. Aulmann S, Schleibaum J, Penzel R, Schirmacher P, Gebauer G, Sinn HP. Gains of chromosome region 3q26 in intraepithelial neoplasia and invasive squamous cell carcinoma of the vulva are frequent and independent of HPV status. JClinPathol. 2008;61(9):1034-7.
56. Borggrefe T, Oswald F. The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell MolLife Sci. 2009;66(10):1631-46.
57. Aithal MG, Rajeswari N. Role of Notch signalling pathway in cancer and its association with DNA methylation. JGenet. 2013;92(3):667-75.
58. Kashofer K, Regauer S. Analysis of full coding sequence of the TP53 gene in invasive vulvar cancers: Implications for therapy. Gynecol Oncol. 2017.
59. Choschzick M, Hantaredja W, Tennstedt P, Gieseking F, Wolber L, Simon R. Role of TP53 mutations in vulvar carcinomas. Int J Gynecol Pathol. 2011;30(5):497-504.
60. Cancer Genome Atlas Research N. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517(7536):576-82.
61. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157-60.
62. McAlpine JN, Leung S, Cheng A, Miller D, Talhouk A, Gilks CB, et al. HPV-independent vulvar squamous cell carcinoma has a worse prognosis than HPV-associated disease: a retrospective cohort study. Histopathology. 2017.
63. Lont AP, Kroon BK, Horenblas S, Gallee MP, Berkhof J, Meijer CJ, et al. Presence of high-risk human papillomavirus DNA in penile carcinoma predicts favorable outcome in survival. Int J Cancer. 2006;119(5):1078-81.
64. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. JNatlCancer Inst. 2008;100(4):261-9.
65. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. ClinCancer Res. 2009;15(22):6758-62.
66. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. IntJCancer. 2007;121(8):1813-20.
67. Lee LJ, Howitt B, Catalano P, Tanaka C, Murphy R, Cimbak N, et al. Prognostic importance of human papillomavirus (HPV) and p16 positivity in squamous cell carcinoma of the vulva treated with radiotherapy. Gynecol Oncol. 2016;142(2):293-8.



APPENDICES

Nederlandse samenvatting
List of abbreviations
List of publications
Dankwoord
Curriculum Vitae

Nederlandse samenvatting

Inleiding

Schaamlipkanker of vulvacarcinoom is een zeldzame gynaecologische tumorsoort die in Nederland jaarlijks bij ongeveer 300 nieuwe patiënten wordt vastgesteld. De laatste jaren is het aantal gevallen van vulvacarcinoom toegenomen, waarschijnlijk door de toegenomen gemiddelde leeftijd van de bevolking en een toename van het aantal infecties met het humaan papillomavirus (HPV), het virus dat ook baarmoederhalskanker kan veroorzaken. Het meest voorkomende tumortype van vulvacarcinoom is het plaveiselcelcarcinoom (90%), uitgaande van de epitheelcellen van de huid van de schaamlippen. Verspreiding van het vulvacarcinoom vindt behalve in de directe omgeving van de tumor soms ook via de lymfebanen plaats, waarbij de lymfeklieren in de liezen het eerste station zijn waar uitzaaiingen worden gevonden.

Behandeling van vulvacarcinoom

De behandeling van eerste keus voor patiënten met vulvacarcinoom is chirurgie. De afgelopen decennia is de chirurgische behandeling van vulvacarcinoom steeds verder ontwikkeld van radicale naar meer behoudende chirurgie. De uitgebreidheid van de chirurgische behandeling van de primaire tumor en de liezen wordt bepaald door de grootte en locatie van de tumor op de vulva. Patiënten met een vulvacarcinoom met een infiltratiediepte van <1 mm hoeven alleen lokaal behandeld te worden, door middel van een zogenaamde radicale lokale excisie van de tumor. Dit houdt in dat de tumor ruim verwijderd wordt, waarbij gestreefd wordt naar een tumor-vrije snijrand van 8 mm of meer. Patiënten met een vulvacarcinoom met een infiltratiediepte van ≥1 mm moeten naast een radicale lokale excisie ook een behandeling van de liezen ondergaan. Indien de tumor op één plek op de vulva is gelokaliseerd en kleiner is dan 4 cm, kan er een schildwachtklierprocedure worden uitgevoerd. De schildwachtklier is de eerste lymfeklier waar tumorcellen naar uitzaaien. De schildwachtklierprocedure bestaat uit het pre-operatief inspuiten van een radioactieve stof en een blauwe kleurstof rondom de tumor op de vulva. Tijdens de operatie kan dan met behulp van het opmeten van de hoeveelheid radioactiviteit en de kleurstof bepaald worden welke lymfklier de schildwachtklier is. Omdat dit het eerste station is waar uitzaaiingen te vinden zullen zijn, hoeft alleen deze klier verwijderd te worden om te onderzoeken of er inderdaad uitzaaiingen zijn naar de lymfeklieren in de lies. Indien er tumorcellen worden gevonden in de schildwachtklier is een uitgebreide operatie van de liezen noodzakelijk. Dit is ook nodig indien de tumor groter is dan 4 cm of indien er meerdere tumoren op de vulva aanwezig zijn. In deze gevallen worden ofwel alle lymfeklieren uit de lies verwijderd (liesklierdissectie), ofwel alleen de vergrote, mogelijk aangedane lymfeklieren verwijderd (een liesklier debulking). Deze uitgebreide liesoperaties zijn geassocieerd met een hoge kans op complicaties, zoals wondinfecties of lymfoedeem in de benen.

Prognose van vulvacarcinoom
De prognose voor patiënten met vulvacarcinoom is over het algemeen goed, met een totale vijfjaars overleving van 70%. De aanwezigheid van lymfkliermetastasen in de liezen heeft een negatieve invloed op de prognose, net als het ontwikkelen van een lokaal recidief na primaire behandeling. Voor vrouwen die een recidief in de lymfeklieren in de liezen ontwikkelen is de prognose zeer slecht; het grootste deel van deze patiënten overlijdt binnen twee jaar aan de gevolgen van dit liesklierrecidief.

Ontstaanswijze van vulvacarcinoom
Vulvacarcinoom kan via twee verschillende routes ontstaan, op basis van de aan- of afwezigheid van HPV. Ongeveer 30% van de vulvacarcinomen ontstaat na langdurige besmetting met HPV en heeft vulvaire high grade squamous intraepithelial neoplasia (HSIL) als voorstadium. Dit type vulvacarcinoom wordt voornamelijk bij jongere vrouwen gevonden en is geassocieerd met roken, promiscuïteit en een niet goed functionerend immuunsysteem.

Het overgrote deel van de vulvacarcinomen (70%) wordt echter niet veroorzaakt door HPV, maar door genetische mutaties en epigenetische veranderingen. Epigenetische veranderingen zijn omkeerbare veranderingen in de functie van genen die optreden zonder wijzigingen in het DNA van de genen. De meest gevonden genetische mutatie bij vulvacarcinomen en voorstadia is een mutatie in het tumor-suppressor gen *TP53*. De HPV-onafhankelijke vulvacarcinomen komen vaker voor bij oudere vrouwen en hebben differentiated vulvaire intraepitheliale neoplasie (dVIN) als voorstadium. Daarnaast bestaat er een sterke associatie tussen lichen sclerosus (LS) en dVIN. LS is een chronische auto-immuun ontsteking die leidt tot verdunning van de huid en uiteindelijk het verdwijnen van de kleine schaamlippen. Vrouwen met LS aan de vulva hebben zo'n 3-5% kans op het ontwikkelen van vulvacarcinoom gedurende hun leven.

Op dit moment is het meeste onderzoek gedaan naar de moleculaire mechanismen die betrokken zijn bij de ontwikkeling van vulvaire HSIL en HPV-afhankelijke vulvacarcinomen. Dit komt mede doordat HPV ook betrokken is bij de ontwikkeling van andere tumorsoorten, zoals baarmoederhalskanker en hoofd-hals kanker.

Inhoud van dit proefschrift
Het doel van dit proefschrift is tweeledig. In het eerste gedeelte hebben we geprobeerd een aantal belangrijke klinische vraagstukken ten aanzien van de behandeling van vulvacarcinoom te onderzoeken. In het tweede gedeelte hebben we onderzoek gedaan om meer duidelijkheid te krijgen over de ontstaanswijze van HPV-onafhankelijke vulvacarcinomen.

In **hoofdstuk 2** hebben we gekeken naar de relatie tussen tumor-vrije snijranden en het krijgen van een lokaal recidief. De tumor-vrije snijrand wordt weergegeven als de afstand in millimeters van de tumor tot de resectierand na bewerking door de patholoog. Op dit moment adviseren de meeste nationale en internationale richtlijnen om te streven naar een minimale tumor-vrije snijrand van 8 mm of meer. Dit wordt geadviseerd om de kans op een lokaal recidief te verkleinen en daarmee de prognose voor patiënten te verbeteren. Om deze marge te bereiken wordt tijdens de operatie ongeveer 15 mm gezond vulvaweefsel rondom de tumor verwijderd. Dit kan in sommige gevallen leiden tot problemen of complicaties, met name als de tumor zich dichtbij de urethra of de anus bevindt. In deze studie hebben wij een meta-analyse van de huidige literatuur verricht en daarbij gevonden dat een tumor-vrije snijrand van <8 mm een bijna 2 keer verhoogd risico geeft op een lokaal recidief. Bij het bestuderen van alle geïncludeerde studies viel echter op dat er veel verschillen waren met betrekking tot de onderzochte patiënten. Ook had vrijwel geen van de studies een duidelijke definitie van een lokaal recidief, iets waar binnen de literatuur veel discussie over bestaat. Deze discussie bestaat mede omdat bekend is dat er ook nieuwe primaire carcinomen in het vulvagebied kunnen voorkomen. Als aanvulling op de meta-analyse hebben wij vervolgens in een cohort patiënten uit het LUMC gekeken naar de relatie tussen de tumor-vrije snijrand en het optreden van een lokaal recidief. In deze cohort studie werd een lokaal recidief gedefinieerd als een nieuwe bewezen tumor die binnen twee jaar na de primaire behandeling en aan dezelfde zijde van de vulva was ontstaan. In deze cohort studie vonden we geen verschil in het aantal lokale recidieven als we patiënten met een tumor-vrije snijrand van <8 mm vergeleken met patiënten met een tumor-vrije snijrand van ≥8 mm (12.6% versus 10.2%). Patiënten waarbij nog tumorcellen in de snijrand werden aangetroffen (een tumor-positieve snijrand) hadden wel een sterk verhoogd risico op een lokaal recidief (30%). Dit betekent dat het sterk de vraag is of een minimale tumor-vrije snijrand van 8 mm inderdaad noodzakelijk is voor het voorkomen van een lokaal recidief. Het lijkt met name essentieel om de tumor volledig te verwijderen, waarbij de marge minder van belang is. Prospectief onderzoek, waarbij een duidelijke definitie voor lokaal recidief wordt gehandhaafd, is nodig om een definitief antwoord op deze vraag te krijgen.

Hoofdstuk 3 van dit proefschrift richt zich op een ander belangrijk klinisch vraagstuk bij de behandeling van vulvacarcinoom, namelijk de behandeling van de lymfeklieren in de lies. Zoals eerder beschreven hebben sommige patiënten een uitgebreide behandeling van de lymfeklieren in de lies nodig, die kan bestaan uit een volledige liesklierdissectie of een debulking van de lymfeklieren, gevolgd door radiotherapie. Adequate behandeling is van groot belang, omdat een recidief in de lymfeklieren in de lies bijna altijd fataal is voor patiënten. Het is echter bekend dat de liesklierdissectie gepaard gaat met een hoog risico op morbiditeit, zoals wondinfecties, wond defecten, lymfoceles en lymfoedeem in

de benen. Vooral lymfoedeem in de benen is een complicatie die leidt tot veel klachten op de lange termijn. Beperktere behandeling van de lymfeklieren in de lies, zoals een debulking, leidt mogelijk tot een afname van de kans op complicaties. In dit onderzoek hebben wij onder andere gekeken naar het verschil in het aantal recidieven in de lymfeklieren in de lies voor patiënten die een liesklierdissectie of een debulking hadden ondergaan. Daarbij vonden we dat het risico op een recidief in de lymfeklieren van de lies voor beide groepen gelijk is (13.3% in de liesklierdissectie groep versus 15.8% in de debulking groep). Patiënten die een recidief in de lymfeklieren in de lies ontwikkelden hadden een 9 keer verhoogd risico op overlijden in vergelijking met patiënten zonder een recidief in de lymfeklieren in de lies. Daarnaast hebben wij het verschil in complicaties tussen de verschillende behandelingen onderzocht. De kans op complicaties was veel lager in de patiëntengroep die een debulking had ondergaan dan in de patiëntengroep die een liesklierdissectie had ondergaan (13.2% versus 53.3%). Deze studie toont aan dat een debulking inderdaad de behandeling van eerste keus zou moeten zijn voor patiënten met een verdenking op uitzaaiingen in de lymfeklieren in de lies.

In **hoofdstuk 4** wordt een samenvatting van de literatuur ten aanzien van de behandeling van recidief vulvacarcinoom gegeven. De kans op het ontwikkelen van een vorm van recidief van vulvacarcinoom ligt tussen de 12-37%. Er zijn verschillende vormen van recidief van het vulvacarcinoom; een lokaal recidief, een regionaal recidief (in de lymfeklieren in de lies) of een recidief op afstand (buiten het kleine bekken). Ondanks verregaande verbeteringen en aanpassingen in de behandeling van vulvacarcinoom is dit aantal niet gedaald in de afgelopen jaren. De behandeladviezen verschillen per vorm van recidief. Voor een lokaal recidief is de eerste keus chirurgische behandeling. Mocht dit niet mogelijk zijn dan is (chemo)radiotherapie een alternatieve behandeling, danwel als definitieve behandeling, danwel om de tumor eerst te verkleinen waarna alsnog chirurgische behandeling kan plaatsvinden. Ook voor een recidief in de lymfeklieren in de lies is chirurgie de eerste behandelkeus. Indien patiënten tijdens de primaire behandeling geen aanvullende radiotherapie hebben ondergaan, wordt geadviseerd om nu wel aanvullend met radiotherapie te behandelen. Ook bij deze patiënten kan (chemo) radiotherapie overwogen worden als chirurgische behandeling niet mogelijk is. Voor recidieven op afstand bestaan alleen palliatieve behandelmethoden. Belangrijk is dat er geen eenduidig advies te geven is voor patiënten met een recidief vulvacarcinoom en dat het behandeladvies dus altijd geïndividualiseerd dient te worden. Behandeling dient dan ook plaats te vinden in een gespecialiseerd ziekenhuis door een multidisciplinair behandelteam.

Hoofdstuk 5 betreft een literatuurstudie waarin een overzicht wordt gegeven van alle tot nu toe beschreven genetische en epigenetische veranderingen in vulvacarcinomen en voorstadia van vulvacarcinomen. Uit deze studie blijkt dat (epi)genetische veranderingen

vaker worden gevonden in HPV-onafhankelijke tumoren dan in HPV-afhankelijke tumoren. Behalve frequent beschreven genetische mutaties in *TP53* is er weinig bekend over genetische veranderingen bij (voorstadia van) vulvacarcinomen. Er zijn enkele studies die epigenetische veranderingen beschrijven, waarbij voornamelijk epigenetische veranderingen in *CDKN2A* werden gevonden.

In **hoofdstuk 6** hebben wij onderzoek gedaan naar de waarde van een immunohistochemische marker, stathmine, om onderscheid te maken tussen laaggradige en hooggradige HPV-afhankelijke voorstadia van vulvacarcinoom. Deze marker wordt ook voor andere laesies gebruikt om dit onderscheid te maken. In dit onderzoek wordt onderscheid gemaakt tussen vulvaire low-grade intraepithelial lesions (LSIL) en HSIL. Dit onderscheid is belangrijk omdat LSIL niet tot vulvacarcinoom leidt, terwijl patiënten met HSIL zonder behandeling een kans van 9-16% hebben op het ontwikkelen van vulvacarcinoom. In deze studie hebben we aangetoond dat stathmine een sensitieve en specifieke biomarker is voor de diagnose vulvaire HSIL en met name gebruikt kan worden in aanvulling op huidige biomarkers indien er twijfel bestaat over de diagnose.
In **hoofdstuk 7** hebben wij met behulp van next generation sequencing (NGS), onderzoek gedaan naar genetische mutaties bij vulvacarcinomen en voorstadia van vulvacarcinomen. In deze studie vonden wij, zoals verwacht, een hoog percentage mutaties in *TP53*, met name in de HPV-onafhankelijke vulvacarcinomen en premaligniteiten. Naast deze mutaties vonden wij ook mutaties in *NOTCH1* en *HRAS*, waarbij genetische mutaties in *NOTCH1* nog niet eerder in de literatuur zijn beschreven. Omdat er veel overeenkomsten waren tussen de mutaties aangetoond bij de voorstadia en de mutaties bij de vulvacarcinomen werd duidelijk dat deze genetische veranderingen al vroeg in de ontwikkeling van vulvacarcinoom een rol spelen. Ook werd duidelijk dat een aanzienlijk deel van de HPV-onafhankelijke vulvacarcinomen géén mutatie had in *TP53* (35%). Het is aannemelijk dat mutaties in *NOTCH1* en *HRAS* bij dit type vulvacarcinoom een belangrijke rol spelen. Deze bevindingen tonen aan dat vulvacarcinomen mogelijk niet in twee, maar in drie verschillende subtypes zou moeten worden ingedeeld. Om aan te tonen of dit ook klinisch van belang is hebben we vervolgens in een groot cohort vulvacarcinomen onderzocht of deze nieuwe indeling leidt tot een verschil in prognose. In dit cohort vonden we een duidelijk betere overleving bij de HPV-afhankelijke vulvacarcinomen in vergelijking met de HPV-onafhankelijke vulvacarcinomen. Daarbij lijkt het erop dat de HPV-onafhankelijke vulvacarcinomen zonder mutatie in *TP53* qua prognose tussen de HPV-afhankelijke vulvacarcinomen en de HPV-onafhankelijke vulvacarcinomen met een *TP53* mutatie in zitten. Deze bevinding moet in een grotere groep worden bevestigd.

De studies beschreven in dit proefschrift hebben tot nieuwe inzichten in zowel de behandeling als de ontstaanswijze van vulvacarcinoom geleidt. In **hoofdstuk 8**

worden de bevindingen in dit proefschrift bediscussieerd en vergeleken met de huidige literatuur. Daarnaast worden mogelijke toekomstige studies beschreven, die hopelijk een opzet zullen zijn voor vervolgonderzoek. Deze ontwikkelingen kunnen bijdragen aan een meer geïndividualiseerd behandelplan voor patiënten met vulvacarcinoom met als uiteindelijk doel een verbetering van de prognose in combinatie met een vermindering van de morbiditeit ten gevolge van de vaak ingrijpende behandeling.

List of abbreviations

AI	Allelic imbalance
CI	Confidence interval
CIS	Carcinoma in situ
CR	Complete response
C-RT	Chemoradiotherapy
CT	Chemotherapy
dVIN	Differentiated vulvar intra-epithelial neoplasia
EBRT	External beam radiotherapy
FACS	Fluorescence-activated cell sorting
FIGO	International Federation of Gynaecology and Obstetrics
FISH	Fluorescence in situ hybridization
H&E	Hematoxylin and eosin
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard Ratio
HRCGH	High resolution comparative genomic hybridization
HSIL	High grade squamous intraepithelial lesion
IFL	Inguinofemoral lymphadenectomy
ISSVD	International Society for the Study of Vulvovaginal Disease
ITC	Isolated tumour cells
KSC	Keratinizing squamous carcinoma
LCR	Ligand chain reaction
LOH	Loss of heterozygosity
LS	Lichen sclerosus
LSC	Lichen sclerosus chronicans
LSIL	Low grade squamous intraepithelial lesion
LVSI	Lymphovascular space invasion
MSI	Microsatellite instability
Ms-PCR	Methylation-specific polymerase chain reaction
NED	No evidence of disease
NGS	Next generation sequencing
NPV	Negative predictive value
NS	Not specified
OS	Overall survival
PCR	Polymerase chain reaction
PD	Progressive disease
PPV	Positive predictive value

PR	Partial response
RE	Restriction endonuclease
RR	Recurrence rate
RT	Radiotherapy
RT-PCR	Real time polymerase chain reaction
S	Surgery
SCCIS	Squamous cell carcinoma in situ
SCCP	Single strand confirmation polymorphism
SD	Stable disease
SN/SLN	Sentinel lymph node
TGGE	Temperature gradient gel electrophoresis
uVIN	Usual vulvar intra-epithelial neoplasia
VAAD	Vulvar acanthosis with altered differentiation
VC	Vulvar cancer
VLSC	Verruciform lichen simplex chronicus
VSCC	Vulvar squamous cell carcinoma
WHO	World Heath Organization

List of publications

Adequate help for patients with cervical cancer? The referral system in Indonesia. A descriptive comparison study in four provinces.
Nooij LS, Sjahjenny S, Schadé E.
Folia Medica Indonesia 2005; 41(1): 36-41.

Trapezius aplasia: Indications for a dual developmental origin of the trapezius muscle.
Nooij LS, Oostra RJ.
Clinical anatomy 2006; 19(6): 547-549.

The reliability of methionine challenge test is not affected by a deficiency in pyridoxal phosphate, folic acid, or cobalamin.
Nooij LS, van Eyck J, Klip H, van de Leur J, Bosma J.
Hypertens Pregnancy 2011; 30(1): 11-7.

The optimal treatment of severe hypertension in pregnancy: update of the role of nicardipine.
Nooij LS, Visser S, Meuleman T, Vos P, Roelofs R, de Groot CJ.
Curr Pharm Biotechnol. 2014; 15(1):64-9.

The long-term immune response after HPV16 peptide vaccination in women with low-grade pre-malignant disorders of the uterine cervix: a placebo-controlled phase II study.
de Vos van Steenwijk PJ, van Poelgeest MI, Ramwadhdoebe TH, Löwik MJ, Berends-van der Meer DM, van der Minne CE, Loof NM, Stynenbosch LF, Fathers LM, Valentijn AR, Oostendorp J, Osse EM, Fleuren GJ, **Nooij LS**, Kagie MJ, Hellebrekers BW, Melief CJ, Welters MJ, van der Burg SH, Kenter GG.
Cancer Immunol Immunother. 2014; 63(2):147-60.

Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: a review of the current literature.
Trietsch MD, **Nooij LS**, Gaarenstroom KN, van Poelgeest MI.
Gynecol Oncol. 2015; 136(1):143-57.

Groin surgery and risk of recurrence in lymph node positive patients with vulvar squamous cell carcinoma.
Nooij LS, Ongkiehong PJ, van Zwet EW, Bosse T, Creutzberg CL, Gaarenstroom KN, van Poelgeest MI.
Gynecol Oncol. 2015; 139(3):458-64.

'See and treat' approach for high grade squamous intra-epithelial cervical lesions.
Nooij LS, Kagie MJ.
European Journal of Gynecological Oncology. 2016; 37 (1): 22-25.

Stathmin is a highly sensitive and specific biomarker for vulvar high-grade squamous intraepithelial lesions.
Nooij LS, Dreef EJ, Smit VT, van Poelgeest MI, Bosse T.
J Clin Pathol. 2016; 69(12):1070-1075.

Tumour-free margins in vulvar squamous cell carcinoma: Does distance really matter?
Nooij LS, van der Slot MA, Dekkers OM, Stijnen T, Gaarenstroom KN, Creutzberg CL, Smit VT, Bosse T, van Poelgeest MI.
Eur J Cancer. 2016; 65:139-49.

Risk factors and treatment for recurrent vulvar squamous cell carcinoma.
Nooij LS, Brand FA, Gaarenstroom KN, Creutzberg CL, de Hullu JA, van Poelgeest MI.
Crit Rev Oncol Hematol. 2016; 106:1-13.

Genomic Characterization of Vulvar (Pre)cancers Identifies Distinct Molecular Subtypes with Prognostic Significance.
Nooij LS, Ter Haar NT, Ruano D, Rakislova N, van Wezel T, Smit VTHBM, Trimbos JBMZ, Ordi J, van Poelgeest MIE, Bosse T.
Clin Cancer Res. 2017; 23(22):6781-6789.

Brief report on 3-weekly paclitaxel carboplatin efficacy in locally advanced or metastatic squamous vulvar cancer.
Amant F, **Nooij LS**, Annibali D, van Rompuy A, Han S, van den Bulck H, Goffin F.
Accepted for publication in Gynecologic and Obstetric Investigation.

Dankwoord

Een proefschrift kan alleen tot stand komen dankzij de hulp van en samenwerking met vele anderen. Graag zou ik een aantal mensen speciaal willen noemen.

Allereerst natuurlijk mijn promotieteam, Prof. Dr. Trimbos, Dr. van Poelgeest en Dr. Bosse.

Beste Baptist, bedankt voor je begeleiding tijdens de totstandkoming van mijn proefschrift. Jouw nuchtere kijk op de stukken heeft mij steeds weer verder geholpen.

Lieve Mariette, bedankt voor de kansen die jij me hebt geboden. Zonder jou had ik nooit op dit punt gestaan. Jouw steun en toewijding aan ons vak en de wetenschap zijn een voorbeeld voor me.

Beste Tjalling, vele uren hebben wij samen achter de microscoop doorgebracht. Bedankt voor je support en de vele kritische discussies die we samen hebben gevoerd.

Daarbij ben ik veel dank verschuldigd aan de familie Rothfusz, die vanuit een legaat geld beschikbaar hebben gesteld voor onderzoek naar vulvacarcinoom.

Prof. Creutzberg en Prof. Smit. Beste Carien en Vincent, veel dank voor het meedenken over de artikelen in mijn proefschrift en jullie kritische noot.

De pathologie afdeling, in het bijzonder Natalja, Michelle, Enno en mijn kamergenoten van p1-40. Natalja, zonder jou was al het laboratoriumwerk me nooit gelukt. Dank voor je geduld, uitleg en de vele uren DNA isoleren, microdissecteren en HPV analyseren. Ook veel dank aan mijn mede-onderzoekers en meiden van de p1-40; Ellen, Inge P, Inge v G, Jolijn, Marthe, en Stephanie. Wat fijn dat ik mijn eindeloze stroom aan vragen steeds aan jullie mocht stellen en dat er naast veel lol ook tijd was om af en toe mijn hart te luchten als er een tegenvaller was. Vanaf nu wordt het 'eat, sleep, work, relax and repeat'.

Mede-onderzoekers van de gynaecologie en verloskunde; Lukas, Sara, Evelien, Kim, Suzanne, Femke, Joost, Ada, Dacia, Mignon en Tess. Dank voor het luisterende oor en de vele gezellige en nuttige koffie en kroket momenten.

De studenten die ik tijdens mijn onderzoek heb mogen begeleiden; Tobias, Michelle, Paulien en Kimberley. Bedankt voor jullie inzet en frisse kijk op het onderzoek.

De afdelingen gynaecologie van het LUMC en Haaglanden MC; alle A(N)IOS, verloskundigen, VA(IOS), verpleegkundigen, secretaressen, doktersassistenten en gynaecologen. En in het

bijzonder mijn opleiders Jan van Lith en Marjolein Kagie. En natuurlijk Marijke Hendriks; dank voor al je hulp tijdens de vele stappen van mijn proefschrift.

Lieve Jiska, Eva, Maartje en Lisette, wat fijn om via de mannen zulke lieve vriendinnen erbij gekregen te hebben. Hopelijk volgen er nog vele etentjes en weekendjes weg!
De mannen van Usque Baugh en friends. Wat een bijzondere vriendengroep zijn jullie! En wat leuk dat ik daar af en toe deel van mag uitmaken. Dank voor de interesse, de steun en het af en toe wegnemen van de zorgen. Ik versla jullie graag nog een keer tijdens de komende pokeravondjes!

Mijn lieve vriendinnen Eva en Laura. Lieve Eef, wij kennen elkaar al zo lang! Wat een feest om jou als vriendin te hebben! Nooit een saai moment als wij samen op avontuur gaan. Alleen vergeten we steeds om ons vriendschapsjubileum te vieren. Laten we nog vele avonturen samen tegemoet gaan en misschien moeten we ons 30-jarig jubileum dan toch maar eens groots gaan vieren! Lieve Lau, ik ken weinig mensen die zo lief en oprecht zijn als jij. Al vanaf het oriëntatiejaar waren wij direct vriendinnen. Hoe fijn was het dat we allebei werden ingeloot voor geneeskunde! Jij doet over het algemeen altijd alles als eerste van ons vriendinnengroepje, behalve promoveren.

Mijn paranimf Ineke. Lieve Ien, vanaf het begin van onze studie hebben we samen al vele beleidsplannen succesvol laten aflopen. Wat een voorrecht om zo'n lieve, gezellige en dierbare vriendin te hebben en wat een eer om jou nu als mijn paranimf te hebben. Laten we nog vele mooie herinneringen gaan maken tot we oud en grijs zijn en gezellig in het bejaardentehuis klaverjastoernooien kunnen houden!

Mijn schoonfamilie, dank voor alle gezelligheid en jullie steun en interesse.

Lieve Coby, wat ben ik blij met jou als mijn schoonmoeder! Dank voor al je wijze woorden en de vele gesprekken. Jij hebt me vaak aan het denken gezet en erdoor heen geholpen als ik weer eens ergens tegenaan liep.

Lief zusje, wat ben ik trots op jou en hoe jij en Nien het allemaal voor elkaar hebben. Ik ben zo blij met jou als mijn zusje en nog gelukkiger dat ik naast mijn leuke neefje nog een nichtje erbij krijg!

Lieve pap en mam, dank voor jullie warmte, gezelligheid, eindeloze steun en interesse. En natuurlijk ook voor de liefdevolle opvang van onze kleintjes. Zonder jullie was mij dit nooit gelukt.

Liefste Tijmen, wie had gedacht dat een flirt tijdens mijn coschap chirurgie ons uiteindelijk op dit punt zou brengen. Het leven met jou is één groot feest, extra opgevrolijkt door onze drie prachtige kinderen. Zonder jouw steun, liefde en relativeringsvermogen was dit me nooit gelukt. Bedankt voor ons fantastische leven samen. Het maakt me extra trots dat jij deze dag naast mij staat als mijn paranimf. Vanaf nu kunnen we gaan genieten van alle mooie dingen die nog gaan komen.

Liefste Jente, Gijs en Mette, jullie maken mijn leven oneindig veel leuker. Ik hou van jullie tot de film en weer terug. En ja, dat is echt nog veel verder dan tot de maan en weer terug.

Curriculum Vitae

Linda Suzanne Nooij was born on February 24th 1980 in Amsterdam and grew up in Almere. In 1998 she graduated from secondary school (VWO) at the Baken College. In 1999 she started with her medicin study at the Academical Medical Centre (AMC) in Amsterdam. During her study she went to Indonesia for a research project from the KWF on public information for prevention of cervical cancer. She combined her internships with a job as a tutor on the department of anatomy in the AMC. In 2006 she attained her medical degree and started working as a physician (ANIOS) in Obstetrics and Gynaecology at the Isala clinics, Zwolle and later at the Haaglanden Medical Centre (HMC), The Hague. In 2009 she started her residency in Obstetrics and Gynaecology at the HMC (Dr. M.J.Kagie) and at the LUMC, Leiden (Prof. Dr. J.M.M. van Lith). In October 2013 she started as a full-time PhD candidate at the departments of Gynaecology and Pathology at the Leiden University Medical Centre (LUMC) (promotor: Prof. Dr. J.B.M.Z. Trimbos, co-promotores: Dr. M.I.E. van Poelgeest and Dr. T. Bosse). The results obtained during this PhD are described in this thesis. During her PhD she presented the results at several national and international congresses. For her presentation during the gynaecongres 2016 in Eindhoven she won the 'Wim Schellekens' award for best short presentation. In July 2016 she continued her residency which she will finish in December 2018. In 2006 she met her husband Tijmen Buddingh in Amsterdam with whom she has three children; Jente (2011), Gijs (2014) and Mette (2017).