OPTIMIZING MANAGEMENT FOR WOMEN WITH VULVAR CANCER

Anne-Floor Willemieke Pouwer

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For reasons of consistency within this thesis, terminology have been standardized throughout the text, and might therefore slightly differ from the original publications.

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Introduction and outline of this thesis

Epidemiology

Vulvar cancer is the fourth most common gynecologic cancer after endometrial, ovarian and cervical cancer. It accounts for 5% of all gynecologic cancers with an incidence rate of 2.5 per 100,000 women [1], with half of the women aged over 70 years at time of diagnosis [2]. The most common histological type is squamous cell carcinoma (SCC), which accounts for over 80% of the cases. Less common histological types of vulvar cancer are basal cell carcinoma, melanoma, adenocarcinoma and extremely rare types such as sarcoma and lymphoma [3]. In the Netherlands, around 385 new patients with vulvar SCC are diagnosed each year [4]. Over the past few decades, the incidence of vulvar SCC is increasing, in both the younger and elderly population [2].

Pathogenesis

There are two different etiologic pathways known for the development of vulvar SCC; non HPV-related and HPV-related. Both pathways have their own associated premalignant lesions [5-8], as shown in Figure 1. The most common type of vulvar SCC is non-HPV related, usually occurring in elderly women in a background of the chronic inflammatory vulvar skin disease lichen sclerosus (LS) [9]. There are strong indicators that differentiated vulvar intraepithelial neoplasia (dVIN) is the direct precursor lesion, but the exact mechanism of progression from LS and dVIN into vulvar SCC is not yet known. The second type of vulvar SCC is caused by a persistent infection with high-risk HPV and associated with the precursor lesion high-grade intraepithelial squamous lesion (HSIL). It primarily affects younger women and is associated with smoking and an immunosuppressive state. The pathogenesis resembles the development of cervical cancer.

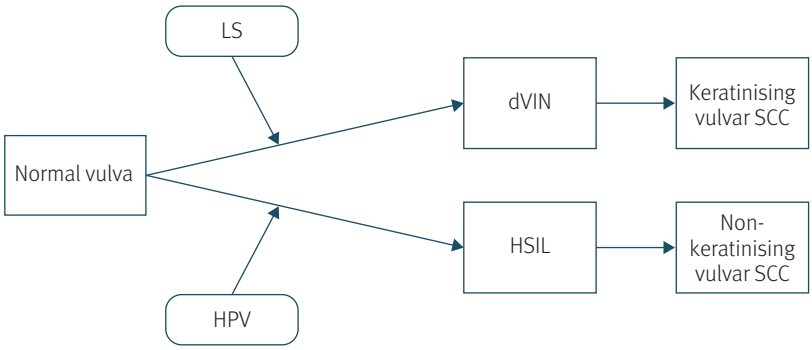

Figure 1 Pathogenesis of vulvar SCC

LS: lichen sclerosus; HPV: human papilloma virus; dVIN: differentiated vulvar intraepithelial neoplasia; HSIL: high-grade squamous intraepithelial lesion; SCC: squamous cell carcinoma

Staging

The dissemination of vulvar SCC may occur by three different routes; lymphogenic, haematogenous and direct extension to adjacent structures. The initial spread occurs usually lymphogenic to the inguinofemoral lymph nodes. As soon as the invasion depth is more than one millimeter, inguinofemoral lymph node metastases can already be present [10]. Direct extension to adjacent structures and haematogenous dissemination is less frequent and generally present in a more advanced stage disease [11, 12]. Vulvar SCC is staged according to the International Federation of Gynecology and Obstetrics (FIGO) 2009, as shown in Table 1. Because prognosis is strongly dependent on the inguinofemoral lymph node status, this is an important part of tumor staging.

Table 1 FIGO stage for carcinoma of the vulva (2009)

FIGO stage		Description
Stage I		Tumor confined to the vulva or perineum, no nodal metastases
	IA	Lesions ≤ 2 cm with stromal invasion ≤ 1 mm*
	IB	Lesions › 2 cm in size or with stromal invasion › 1 mm
Stage II		Tumor of any size with adjacent spread to the perineal structures (1/3 lower urethra, 1/3 lower vagina, or the anus) and no nodal metastases
Stage III		Tumor of any size confined to vulva or with adjacent spread to the perineal structures (1/3 lower urethra, 1/3 lower vagina, or the anus) with positive inguinofemoral lymph nodes
	IIIA	(i) with 1 lymph node metastasis ≥5 mm, or (ii) with 1 or 2 lymph node metastases ‹ 5 mm
	IIIB	(i) with 2 or more lymph node metastases ≥5 mm, or (ii) with 3 or more lymph node metastases ‹5 mm
	IIIC	With positive lymph nodes with extracapsular spread
Stage IV		Tumor invades other regional (2/3 upper urethra, 2/3 upper vagina) or distant structures
	IVA	(i) Tumor with spread into upper urethra/vagina, bladder, rectal mucosa, bone or fixed to pelvic bone (ii) Tumor with fixed or ulcerated inguinofemoral lymph nodes
	IVB	Any distant metastases, including pelvic lymph nodes

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

Adapted from the FIGO classification from Pecorelli et al. (2009)[13].

Treatment

The method of treatment for vulvar SCC depends on the extent of the disease and does not differ per etiologic pathway. The cornerstone of treatment is surgery which consists of wide local excision (WLE) with or without groin surgery depending on the depth of invasion of the vulvar SCC. For WLE of the vulvar tumor, it is recommended to obtain surgical tumor free margins of at least one to two centimeters [14-18]. The pathologic tumor free margin of › 8 mm is considered standard since years. Currently there is an ongoing debate on the pathologic tumor fee margin, resulting in the ESGO guideline without strict advice.

In patients with primary microinvasive vulvar SCC (depth of invasion ≤1 mm) and a diameter of ≤2 cm, treatment entails WLE of the tumor without groin surgery as the risk for groin lymph node metastases is very low (‹ 1%) [19, 20]. In patients with primary macroinvasive vulvar SCC (depth of invasion › 1 mm), about 20-30% of the patients without suspicious lymph nodes at clinical examination appear to have lymph node metastases [21-23]. There is no non-invasive method with a high enough negative predictive value to accurately rule out the presence of inguinofemoral lymph node metastases [10]. It should be kept in mind that the consequences of not detecting groin metastases are significant because these patients have a 5-year survival of 0-10% [24]. Therefore, groin surgery is indicated in all patients with a macroinvasive vulvar SCC and consists of a sentinel lymph node (SLN) procedure and/or inguinofemoral lymphadenectomy (IFL). In patients with a unifocal tumor measuring ‹ 4 cm without suspicious lymph nodes in the groin a SLN procedure can be performed and IFL can be safely omitted [25, 26]. In patients not eligible for the SLN procedure (tumor measuring › 4 cm and/or multifocal tumor and/or suspicious lymph nodes in the groin) or if the SLN is positive, IFL is indicated.

Adjuvant treatment

Local re-excision is indicated if the pathologic margin is tumor positive. Radiotherapy to the vulva is recommended if the pathologic margin is tumor positive re-excision is not possible [15-18]. In patients with a close margin, either adjuvant re-excision or radiotherapy to the vulva is considered, or close follow up is performed.

Adjuvant radiotherapy to the groin is performed in patients with one inguinofemoral lymph node metastasis with extranodal growth and/or more than one lymph node metastases without extranodal growth.

Treatment associated morbidity

The treatment of vulvar SCC is associated with high complication rates, especially in the groin(s). The past few decades, several adjustments of the surgical technique were implemented in order to reduce the morbidity without compromising the prognosis. The most important adjustments were the replacement of the classic radical

vulvectomy with 'en bloc' bilateral IFL by WLE of the tumor combined with IFL by separate incisions. In addition, the introduction of the SLN as a safe alternative for IFL in a selected group of patients with primary vulvar SCC significantly reduced the postoperative morbidity [25, 26].

Since the introduction of the SLN, the number of patients with an indication for IFL decreased. However, IFL is finally indicated in around half of the patients with primary vulvar SCC based on multifocal tumors, tumors › 4 cm or a positive SLN and in all patients with local recurrent disease if IFL has not been performed previously [27]. IFL has significant short- and long-term complications of the groin, which are reported in up to 85% of the patients [23, 27, 28]. Wound breakdown, wound infection and formation of lymphoceles are the most documented short-term complications. The development of lymphedema and (recurrent) cellulitis or erysipelas are the most documented long-term complications. Known risk factors for short- and long-term complications are older age, diabetes, 'en bloc' surgery and higher drain production on the last day of drain in situ [27]. Studies concerning the postoperative care including optimal drain management in relation to postoperative morbidity are lacking.

Follow-up

The follow-up after surgical treatment for primary vulvar SCC is consensus based rather than evidence based, and prospective studies are lacking. As the treatment of a local recurrence is largely dependent on either further excision or radiotherapy, follow-up is performed to detect locoregional recurrences as early as possible to achieve better survival rates preferably with limited morbidity. A study of Oonk *et al.* showed that local recurrences detected during a routine follow-up meeting were significantly smaller compared to recurrences detected at interval meetings scheduled because of complaints [29]. Smaller recurrences might lead to less extensive surgery.

Worldwide, guidelines recommend different follow-up regimens; advised regimens range between three- and six-monthly visits during the first two years, six- until 12-monthly for the third, fourth and fifth year and all recommend annually thereafter [15-18]. However, none of these follow-up regimens is individualized based on the performed treatment and/or risk of developing recurrent disease.

Prognosis

The inguinofemoral lymph node status at initial diagnosis is of critical prognostic importance for patients with vulvar SCC [11, 12, 30-32]. The five-year survival for early stage vulvar SCC is 70-93% and decreases to 25-41% with the presence of lymph node metastases [33]. In patients suffering from a groin recurrence, prognosis is very poor with a 5-year survival rate of 0-10% [24].

Local recurrent disease

In patients treated for primary early-stage squamous cell carcinoma of the vulva, local recurrence is reported in up to 40% during the first ten years after treatment and is associated with significantly decreased disease-specific survival [34]. Treatment of a local recurrence consists of WLE combined with IFL if not performed as part of the treatment for the primary vulvar SCC [10]. As a consequence, patients with a local recurrence after an earlier SLN procedure, undergoing an IFL do not perceive the benefits of the SLN procedure anymore, in terms of a reduction in morbidity.

OUTLINE OF THIS THESIS

The oncogenesis of non HPV-related vulvar SCC remains to be unravelled. DVIN is mostly found in a background of LS and can give rise to vulvar SCC, but genetic evidence is currently limited. To study the genetic relationship between these lesions, we compared genomic abnormalities of vulvar SCC lesions with paired dVIN and/or LS lesions in Chapter 2. The depth of invasion predicts biological behavior of vulvar SCC and determines the need for groin surgery. A high interobserver agreement is important to support the prognostic value for the presence of lymph node metastases. In Chapter 3 we assessed the interobserver agreement in the classification of the depth of invasion and identified pitfalls in the assessment of the depth of invasion in order to improve the inter-observer agreement between pathologists.

In patients treated for early-stage vulvar SCC, local recurrences are reported in up to 40% ten years after primary treatment. We summarized the current knowledge on the incidence and prognostic factors for local recurrent disease in Chapter 4 and describe in Chapter 5 the results of our multicenter study to assess the influence of tumor and/or precursor lesion free pathologic margins, clinicopathologic and tumor characteristics on local recurrence rate.

In Chapter 6, we focused on the morbidity associated with IFL in patients with vulvar SCC. We created an up-to-date summery of the current literature on surgical techniques and peri- and postoperative care in relation to morbidity. In Chapter 7 we describe the results of our nationwide prospective study, which assessed the feasibility and the incidence of postoperative complications after volume-controlled versus short drainage of the groin. Subsequently, we focused on the surgical technique of IFL with the aim to further reduce morbidity after IFL. In Chapter 8 we present the results of our randomized controlled trail comparing two surgical techniques for IFL: LigaSure and conventional performance of IFL.

To optimize the follow-up in patients treated for early-stage vulvar SCC, we describe in Chapter 9 the efficacy of the addition of an ultrasound of the groin to the routine follow-up during the first two years in patients with a negative sentinel lymph node. The general discussion in Chapter 10 gives an overview of the findings presented in this thesis, and based on these findings future perspectives in the management of vulvar SCC are discussed.

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Clonal relationship between lichen sclerosus, differentiated vulvar intraepithelial neoplasia and non human papillomavirus (HPV) related vulvar squamous cell carcinoma

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ABSTRACT

Differentiated vulvar intraepithelial neoplasia (dVIN) is mostly found in a background of lichen sclerosus (LS) and can give rise to vulvar squamous cell carcinoma (SCC), but genetic evidence is currently still limited. To study the genetic relationship between these lesions, we compared genomic abnormalities of vulvar SCC lesions with paired dVIN and/or LS in twelve patients. High-resolution genome-wide copy number analysis was performed for six vulvar SCC samples and three paired dVIN samples. Copy number alterations were identified in all vulvar SCC samples. One dVIN lesion presented with three copy number alterations that were preserved in the paired vulvar SCC sample, including a 11q23 high-level amplification. Subsequently, we sequenced *TP53*, a recurrently mutated driver in vulvar SCC, in two patients with acquired homozygosity at 17p and six additional patients, and identified mutations in five cases. Targeted deep-sequencing in the available precursor lesions revealed that all five mutations were traced back in the dVIN (n=5) or the LS (n=1) with frequencies ranging from 3 to 19%, suggesting that mosaic subpopulations of cells in the LS and dVIN lesions gave rise to the vulvar SCC. Our data provide genetic evidence for a clonal relationship between vulvar SCC and dVIN or LS.

INTRODUCTION

Vulvar cancer is the fourth most common cancer affecting the female genital tract and has an increasing incidence of 2.5/100.000 women per year [1]. Approximately 80% of all vulvar cancers are of squamous origin and can develop following two different pathways [2, 3]. The minority of vulvar squamous cell carcinomas (SCC) is caused by a persistent infection with high-risk human papillomavirus (HPV), inducing the precursor high grade squamous intraepithelial lesion (HSIL); the oncogenesis of HPV-related vulvar SCC resembles the development of cervical cancer. The second and most common type of vulvar SCC is non HPV-related and accounts for over 80% of all vulvar SCC [4, 5]. These carcinomas arise in a background of the chronic inflammatory vulvar skin disease Lichen Sclerosus (LS) and/or differentiated vulvar intraepithelial neoplasia (dVIN). The exact role of LS and dVIN in the development of vulvar SCC is not yet known, but patients with LS have a lifetime risk of 4-5% to develop vulvar SCC [6].

There is clinical evidence that dVIN is the precursor lesion of the non HPV-related pathway [7]. We showed that of all VIN lesions diagnosed in the Netherlands between 1992 and 2005, only a minority were dVIN in comparison to HSIL; apparently dVIN is rarely found as a solitary lesion [8]. Remarkebly, dVIN is present around the majority of the non HPV-related vulvar SCCs [4, 5]. This discrepancy, low incidence of solitary dVIN lesions but high incidence of dVIN adjacent to vulvar SCC, can be explained by the fact that the diagnosis of dVIN is clinically and histopathologically difficult [9] with an assumed short intraepithelial phase, which suggests that dVIN is possibly suffering from underdiagnosis [10]. On the other hand, it has been hypothesised that dVIN is a border phenomenon of vulvar SCC [11]. Though the oncogenesis has not been fully clarified, there are strong indications that dVIN is a precursor lesion; in recent years the incidence of solitary dVIN is increasing [8] and dVIN is more often found in revised biopsies previously diagnosed as LS in patients that later developed vulvar SCC [10]. Furthermore, first indications for a genetic correlation between vulvar SCC, dVIN and LS were reported by Nooij *et al.*[12].

Few studies have investigated genetic abnormalities in non HPV-related vulvar SCC, and showed frequent occurrence of *TP53* mutations [13-15]. The upcoming techniques to search for genetic changes are promising to provide more information on the aetiology of (pre)malignant vulvar lesions. However, the literature on this topic is limited in vulvar (pre)malignancies. In theory, tumours arise from a multistep process of accumulated genetic alterations [16]. The identification of chromosomal regions most frequently affected by copy number alterations or sequence mutations may be relevant for determining the relationship between LS, dVIN and vulvar SCC.

We hypothesise that LS and dVIN are lesions already showing early neoplastic alterations which are also present in vulvar SCC together with other accumulating events. In order to provide information on the mutations that can be found in vulvar

SCC and to provide more evidence for a clonal relation between LS, dVIN and vulvar SCC, we aimed to determine genetic abnormalities in vulvar SCC of 12 patients and backtrack these abnormalities in the dVIN and LS precursor lesions.

METHODS

Tissue samples

Twelve patients with a primary vulvar SCC who were primary surgically treated between 2008 and 2014 in the Radboud university medical center Nijmegen, the Netherlands, were included. Inclusion criteria were: a history of LS, presence of dVIN in the surgical excision specimen, non HPV-related tumour, and no previous radiotherapy or chemotherapy. Clinical data of included patients was retrieved.

To select for non HPV-related VSSCs, we used the $p16^{INK4A}$ expression of the tumour in combination with HPV status and morphologic criteria on hematoxylin and eosin (H&E) stained slides. Tumours were considered as non HPV-related if p16 staining was negative, dVIN and LS were present, independent of HPV status, or if the HPV status was negative in combination with the presence of dVIN and LS.

For this study, coded residual tissue was used, which was retrieved during regular treatment. According to the Dutch law, no specific patient approval is necessary for the use of this material. This study was approved by the local ethical committee (CMO Regio Arnhem-Nijmegen; registration number 2015-1808) and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies (htpp://federa.org).

Microdissection and DNA extraction

The haematoxylin & eosin stained (H&E) slides of the surgical excision specimen were reviewed by an expert gynaecopathologist; areas with LS, dVIN and vulvar SCC were identified. Formalin-fixed paraffin-embedded (FFPE) blocks were retrieved and re-cut at 5 µm. The last re-cut slide was H&E stained and compared with the original slide in order to confirm that the lesion was still present. Tumour cells were collected through removal of vulvar SCC and dVIN tissue by scraping with a clean scalpel from several unstained slide sections. DNA was isolated with TET-lysis buffer (10 mmol/L Tris-HCl, pH 8.5; 1 mmol/L EDTA, pH 8; 0.1% Tween-20) containing 5% Chelex-100 (Bio-Rad, Hercules, CA). Protein digestion was performed by adding 20 µL of proteinase K to each sample following incubation at 56°C for 48 hours. Fresh proteinase K of 10 µL was added after 24 hours. Next, DNA was denatured by heat inactivation at 95°C for ten minutes. The samples were centrifuged for ten minutes at 14.000 rpm (RT) and measured by Picogreen measurements (Invitrogen, Carlsbad, CA, USA).

Copy number profiling

Genome-wide copy number profiling was performed on DNA from six paired dVIN and vulvar SCC lesions using FFPE-compatible Affymetrix Oncoscan arrays (OncoScan FFPE Express v.2; Affymetrix, Santa Clara, CA, USA), according to the protocol provided by the manufacturer [17-19]. The data that passed quality control (MAPD value ≤ 0.6) were then analyzed using Nexus Copy Number software Edition 7 (Biodiscovery, El Segundo, CA, USA) with NCBI build 37 of the human genome. The SNP-FASST2 Segmentation Algorithm was used. The significance threshold was set at 5.0E-7 with a minimum of 250 probes and a maximum contiguous spacing of 1000 kb to define a segment. Thresholds for copy number gains and losses were set to 0.3 and -0.3, respectively. Thresholds for high-level amplifications and homozygous losses, indicating greater than a single copy number change were set at 1.2 and -1.2 respectively. The homozygous frequency threshold was set at 0.85 and the homozygous value threshold was set at 0.8 with a minimum loss of heterozygosity (LOH) requirement of 500 kb. The heterozygous imbalance threshold was set at 0.4. Allelic imbalance and LOH was only scored when larger than 15 Mb, and adjacent sequential calls of allelic imbalance and LOH were merged as one region upon visual inspection of the copy number and B-allele frequency plots. To allow comparison of vulvar SCC copy number profiles with those of the dVIN samples, which were of poorer quality, we performed smoothening of the dVIN probe intensity values using a 15x running smoothing of the median (R package). Copy number plots of vulvar SCC and smoothened dVIN data per chromosome were made using GenomeGraphs library 1.28.0 [20].

Targeted sequencing of TP53 using molecular inversion probes

For the targeted enrichment of genomic *TP53* sequences we used single molecule Molecular Inversion Probes (smMIPs), which are 80-nt oligonucleotides with two targeting arms at the extreme ends that bind the genomic template in a strand specific manner, allowing strand specific amplification and double tiling. Each probe contains an 8-nt random molecular tag that enable the identification of unique captures from the generated sequencing reads, which is essential in the context of gDNA with low quality/quantity, and can correct for amplification bias [21]. In addition, the molecular tag guided assembly into consensus reads diminishes the number of false positive variants due to PCR and sequencing artefacts and thus allows accurate detection of low-frequency variants. In total, 50 probes were designed that target >95% of coding and splice-sequences (-/+5 nt flanking exon-intron boundaries) in *TP53*. An equimolar pool of these 50 probes was tested and optimized on gDNA controls prior to sequencing of the samples (Supplementary Table S1). To prevent allelic drop-out at sites containing SNPs, identical smMIPs were designed for the major and minor alleles (referred to as 'a' and 'b'). Targeted capture with smMIPs was performed as previously described [21 22]. Shortly, 100 ng of genomic DNA in a pool containing 5'-phosphorylated smMIPs

were annealed overnight to capture the targeted sequences of interest. After annealing, the gaps were closed in a 5'->3' extension reaction, followed by a ligation step to create circular DNA. Uncircularised probes and remaining linear genomic DNA were removed by exonuclease treatment. Barcoded Illumina primers were incorporated in the PCR reaction, following amplification of all pooled samples. Subsequently, the barcoded library was sequenced using the Illumina NextSeq500 system, with 2 × 150-bp paired-end reads. We used SeqNext software (JSI Medical Systems; version 4.2.0) to map and align the reads, create unique consensus reads from PCR duplicates to minimise sequencing errors, and perform variant calling (relevant settings are described in [23]). Identified mutations were validated by Sanger sequencing in LS, dVIN and vulvar SCC samples from the same patient (primers available upon request).

RESULTS

Patient samples

We included twelve patients with a non HPV-related vulvar SCC in this study, of which tissues of six patients were included for whole genome copy number profiling. For each of the twelve included patients, a paired dVIN and vulvar SCC sample was available, for two of them also an LS sample was available for analyses (See Table 1). The median age of our patients was 59 years (range 47-83). Eight patients (67%) had FIGO stage IB and four (33%) stage III disease. Five vulvar SCCs were well differentiated (42%), three moderate differentiated (25%) and four poorly differentiated (33%).

Chromosomal alterations in vulvar SCC

The dVIN and vulvar SCC samples of six patients were hybridised to Oncoscan arrays. All six vulvar SCC samples generated copy number profiles that passed quality control and could be further analyzed (See Supplementary Figure 1). An overview of the distribution and number of gains and losses per patient per sample are displayed in Figure 1 and Table 2. In vulvar SCCs, we identified a total of 94 copy number alterations (Table 2 and supplementary Table S2). These alterations consisted of 55 losses and 39 gains, including 7 high-level amplifications. Overall, eleven genomic regions were affected by copy number alterations in three or more vulvar SCCs (see Supplementary Table S3).

The most frequently found alteration was loss of chromosome 8p, present in all vulvar SCC, followed by gain of chromosome 8q in five of six vulvar SCCs, gain of chromosome 7p and loss of chromosome 18q, both present in four of six vulvar SCCs (Figure 1 and supplementary Table S3).

As shown in Table 3, seven high-level amplifications were seen in three of six vulvar SCCs. Five of these encompassed large genomic regions (37-45 Mb), affecting many

Table 1 Patient demographics, clinical characteristics, and sample availability

Patient	Age	FIGO stage	Samples[1]	Differentiation tumour
1	83	IB	01VSCC 01dVIN 01LS[2]	Poor
2	81	IB	02VSCC dVIN*	Well
3	54	III	03VSCC 03dVIN 03LS[2]	Moderate
4	57	III	04VSCC 04dVIN*	Moderate
5	50	IB	05VSCC 05dVIN*	Well
6	47	IB	06VSCC 06dVIN	Well
7	63	IB	07VSCC[2] 07dVIN[2]	Moderate
8	55	IB	08VSCC[2] 08dVIN[2]	Poor
9	75	III	09VSCC[2] 09dVIN[2]	Poor
10	61	IB	10VSCC[2] 10dVIN[2]	Well
11	78	III	11VSCC[2] 11dVIN[2]	Poor
12	55	IB	12VSCC[2] 12dVIN[2]	Well

FIGO stage: stage of tumour classified by the FIGO classification 2009; *sample did not pass quality control, [1]The VSCC and dVIN lesion of each patient was collected at the same point of time. [2]samples were only used for targeted sequencing of *TP53*.

genes. The others were smaller (1-8 Mb) in size and contained known oncogenes including *EGFR* (7p21.1), *FGFR1* (8p11.23), *IL11-RA* (9p13), and *YAP1* (11q22.1).
Furthermore, 12 regions of allelic imbalance were identified, representing copy neutral homozygosity. These regions are known as acquired uniparental disomy, and frequently contain mutations in tumour suppressor genes that have become homozygous as a result of mitotic recombination. The regions were not completely homozygous, suggesting them to be present in a subset of the sample because of the presence of normal cells and/or tumour heterogeneity (supplementary Table S3).

Interestingly, chromosome 17p encompassing, among other genes, the tumour suppressor gene *TP53*, was affected by acquired uniparental disomy in three vulvar SCCs (Supplementary Table S3). Targeted sequencing of the *TP53* gene using molecular inversion probes in two vulvar SCC samples for which sufficient material remained indeed identified mutations in patient 1 (c.596G>T, p.Gly199Val) and patient 3 (c.833C>A, p.Pro278His). Both mutations were confirmed by Sanger sequencing.

Table 2 Overview of gains, losses and acquired uniparental disomy events per patient per vulvar SCC sample

Patient	Total CNAs	Gains[1]	Losses	High-level amplifications	aUPD
1	21	11	10	2	2 (10p, 17p)
2	17	9	8	3	2 (5, 12q)
3	33	7	26	2	2 (11q, 17p)
4	9	4	5	0	4 (3q, 6p, 9p, 9q)
5	13	8	5	0	2 (13q, 17p)
6	1	0	1	0	-
Total	94	39	55	7	12
Average	15.7	6.5	9.2	1.2	2

[1]including high-level amplifications
Abbreviations=CNA: Copy number alteration; aUPD: acquired uniparental disomy

Clonal relationship between vulvar SCC and dVIN

Next, we analysed whether the chromosomal abnormalities detected in the vulvar SCC could already be detected in the paired dVIN samples. Three dVIN samples (patients 1, 3, and 6) passed quality control and could be further analysed. No copy number alterations were found the dVIN samples of patients 1 and 6. In contrast, three copy number alterations were detected in the dVIN sample of patient 3; a 54-year old female with a unifocal lesion of 2.5 cm on the left labium minus localized 1.5 cm left from the clitoris. Histopathological examination showed that the dVIN was located in the immediate surroundings of a moderately differentiated vulvar SCC with presence of lymphovascular invasion, an invasion depth of 9.5 mm and a sentinel node with isolated tumour cells (Figure 2).

The copy number abnormalities in this dVIN included an amplification of 11q22 and a deletion at the telomeric region of 12q. Furthermore, chromosome 14 showed a higher median probe level, suggesting a gain of this entire chromosome. All these three copy

Figure 1 Copy number alteration (CNA) profiles of six vulvar squamous cell carcinoma (SCC) patients.
Gains are indicated by the blue bars, and losses are shown by the red bars. Dark colors represent multi-copy gains and losses.

number alterations were also detected in the paired vulvar SCC sample (Supplementary Table S2). Detailed comparison between the dVIN and vulvar SCC showed that the boundaries of the 11q22 amplification appeared identical, suggesting that this lesion is indeed preserved from dVIN (Figure 3A and 3B). The 12qter deletion was found to be larger (~1.9 Mb; 197 probes) in the dVIN sample compared to vulvar SCC (Figure 3C), but the quality and amount of available DNA did not allow us to reveal whether this difference was due to a technical artefact or it indicates that the 12qter deletions have arisen independently in dVIN and vulvar SCC. Importantly, the other copy number alterations detected in vulvar SCC (five gains and 25 deletions), including an 8q22-q24.3 amplification (Figure 3D and Table 3) could not be detected in the paired dVIN sample. In conclusion, the shared copy number alterations between the dVIN and vulvar SCC in patient 3 provide genetic evidence for a clonal relationship between dVIN and vulvar SCC.

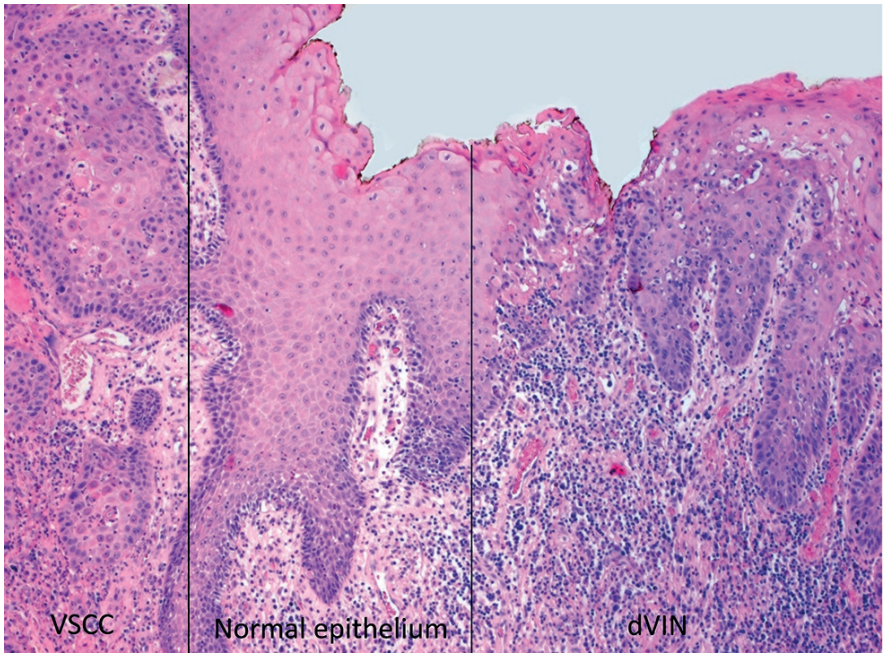

Figure 2 Histological overview of the epithelium of patient three; the area on the left side shows vulvar squamous cell carcinoma (SCC), the area on the right side differentiated vulvar intra-epithelial neoplasia (dVIN).

Micro-dissection was performed in these areas, there is a distance of 2 mm (normal epithelium) between both areas. (original magnification x50)

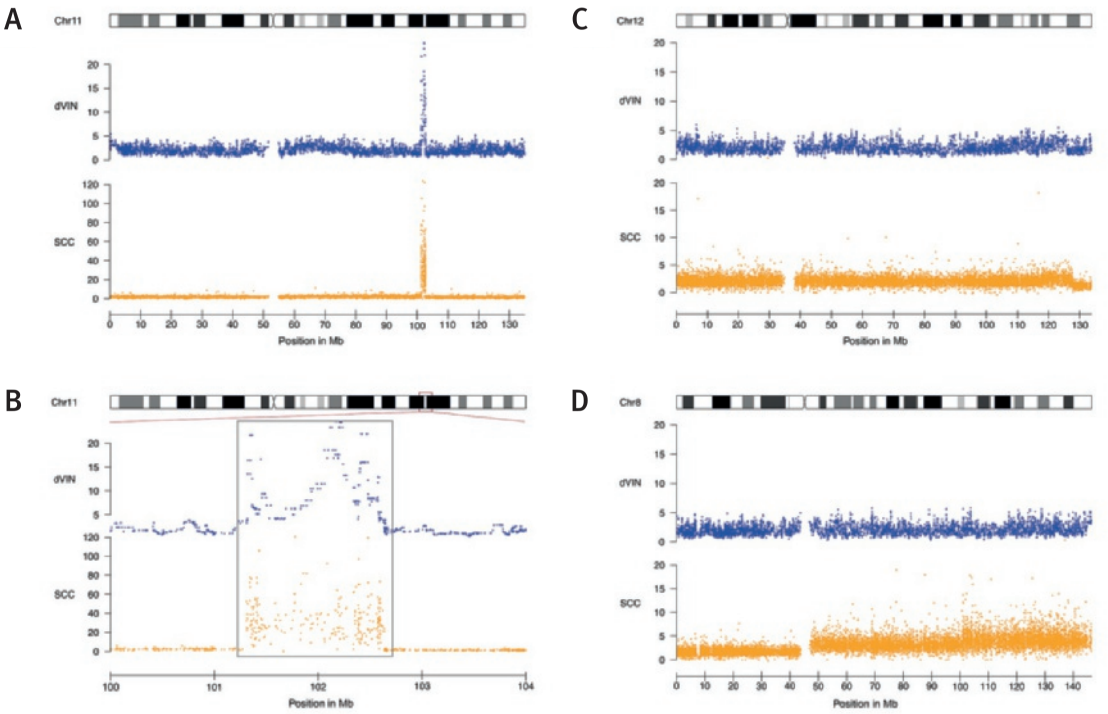

Figure 3 Shared and different copy number abnormalities in differentiated VIN (dVIN) and vulvar squamous cell carcinoma (SCC) of patient three.

Shown are the high-level amplification on chromosome 11 (A and B) and the deletion of 12q-ter (C), which is shared between the two samples, and the copy number gains on chromosome 8, which is absent in dVIN (D). Detail of the amplification on chromosome 11 (B) shows that its boundaries are identical between the two samples.

Table 3 High-level amplifications in vulvar SCC samples

Sample	Chromosome	Start	End	Length (Mb)	Cytoband	Number of genes	Candidate genes
01VSCC	chr09	0	37.625.771	37,63	p24.3 - p13.2	many	
01VSCC	chr13	71.643.837	74.278.611	2,63	q21.33 - q22.1	6	*DACH1, MZT1, BORA, DIS3, PIBF1, KLF5*
02VSCC	chr07	51.965.546	59.863.318	7,90	p12.1	21	*EGFR*
02VSCC	chr08	34.606.454	39.007.911	4,40	p12 - p11.22	22	*FGFR1*
02VSCC	chr09	32.862.231	37.441.137	4,58	p13.1 - p13.2	71	*IL11-RA, DCTN3*
03VSCC	chr08	100.841.341	146.364.022	45,52	q22.2 - q24.3	many	
03VSCC	chr11	101.221.509	102.634.411	1,41	q22.1 - q22.2	12	*YAP1, BIRC2, MMP7*

Subclonal TP53 mutations in dVIN and LS

A clonal relationship between vulvar SCC and dVIN or LS can easily be missed if the alteration is present in only a subset of the cells. Since subclonal copy number alterations are difficult to detect, we next focused on point mutations using smMIP-based targeted deep-sequencing. This technology allows clustering of sequence reads derived from the same template to create so-called single molecule consensus reads (smc reads) [21, 24] which have much lower error rates, hence creating higher specificity for detecting low-level mosaic mutations. We performed smMIP-based sequencing of vulvar SCC samples from six additional patients for the *TP53* gene as this gene is known as a recurrently mutated driver of vulvar SCC.

Next to the mutations already found in patients 1 and 3, we identified three additional *TP53* mutations in the vulvar SCCs of patients 7, 9 and 11 (Table 4). Next, we established whether these five *TP53* mutations could be traced back in paired dVIN and LS samples. In all five patients, the *TP53* mutations detected in the vulvar SCC sample were also present in dVIN with variant allele frequencies ranging from 3 to 19% (Table 4). In patient 1, the mutation was detected by only a single smc-read, likely due to the limited availability of material, which makes an estimate of the mutant allele frequency rather inaccurate. Interestingly, however, this mutation was also detected in the LS sample (6% of the reads), a finding that could be confirmed by Sanger sequencing (Figure 4). In conclusion, we here demonstrate that in all five *TP53*-mutated vulvar SCCs, the mutation could be detected at subclonal levels in dVIN or LS, thereby confirming a clonal relationship between these lesions in the respective patients.

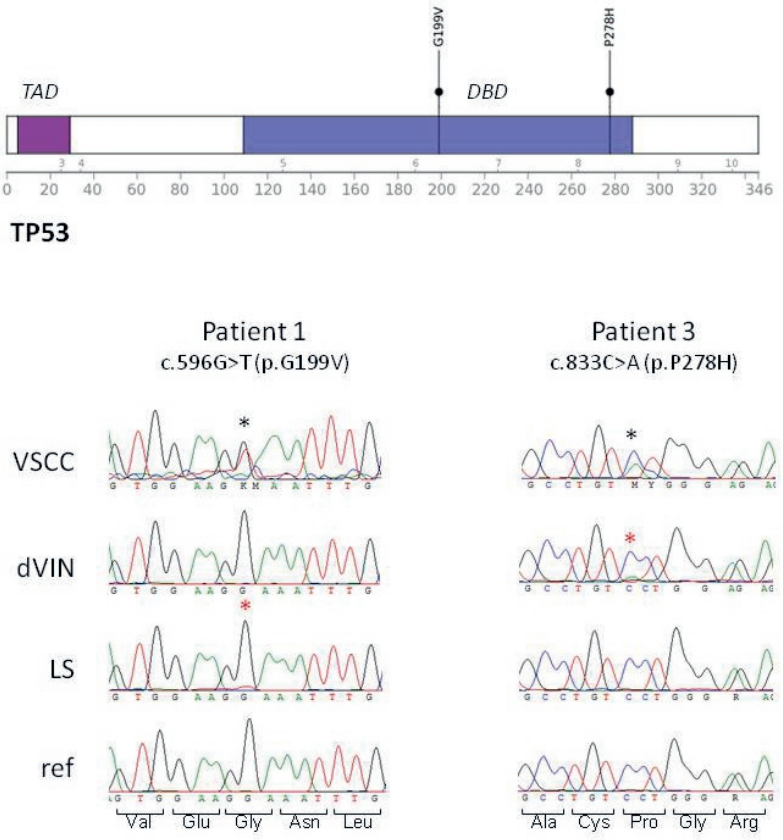

Figure 4 Detection *TP53* mutations in two vulvar squamous cell carcinoma (VSCC) samples and backtracking in differentiated vulvar intra-epithelial neoplasia (dVIN) and lichen sclerosus (LS).

Top panel shows the TP53 protein with the transactivating domain (TAD) and the DNA-dinding domain (DBD) and the location of the two mutations. Below that are sequence chromatograms of each mutation in the respective samples. Mutations are indicated by a black (clonal) or red (subclonal) asteriks.

Table 4 TP53 mutations detected by molecular tagged MIP capture in the VSCC, dVIN and/or LS.

Patient	Sample	Mutation[1]	smMIPs[2]	Read depth	Unique reads	Unique variant reads	Mutant allele frequency
1	01VSCC	c.596G>T	2	30,436	348	142	41%
	01dVIN	c.596G>T	2	4,490	32	1[3]	3%[3]
	01LS	c.596G>T	2	21,760	114	7	6%
3	03VSCC	c833C>A	3	105,912	1,024	714	70%
	03dVIN_a	c833C>A	3	77,646	320	10	3%
	03dVIN_b	c833C>A	3	7,138	36	6	17%
	03LS_a	c833C>A	3	6,038	24	0	0%
	03LS_b	c833C>A	3	2,402	12	0	0%
7	07VSCC	c.130delA/ p.Met44Cysfs*79	2	18,468	55	24	44%
	07dVIN	c.130delA/ p.Met44Cysfs*79	2	36,979	86	17	19%
9	09VSCC	c.-29+5G>A/-	2	20,634	111	49	44%
	09dVIN	c.-29+5G>A/-	2	67,416	143	7	5%
11	11VSCC	c.527G>T/ p.C176F	2	17,100	159	32	20%
	11dVIN	c.527G>T/ p.C176F	2	78,788	211	33	15%

[1]annotation based on transcript (ENST00000269305) and protein (NP_000537.3). [2]number of independent smMIPs (small molecule Molecular Inversion Probes) that capture the mutation in a tiling manner. [3]Variant did not pass variant calling thresholds, but was detected by visual inspection of the data.

DISCUSSION

We investigated genomic alterations of non HPV-related vulvar SCC lesions and patient-matched dVIN lesions using genome-wide copy number profiling and targeted sequencing of *TP53*. We found that gains of 7p and 8q and losses of 8p and 18q were found in most vulvar SCC lesions, and acquired uniparental disomy was common, including for chromosome 17p. In one patient, three of the 33 copy number alterations found in the vulvar SCC sample were also detected in the paired dVIN sample including a high-level amplification on 11q22 and a deletion of 12qter. Furthermore, in five of the 12 patients (42%) we identified a *TP53* mutation in a cellular subset of LS or dVIN, which was shared with the paired vulvar SCC. We therefore conclude that, at least in five patients, the vulvar SCC originate from single precursor cells in which a subset of the genetic alterations, possibly driving premalignant events, were already present in the dVIN and/or LS stage, after which additional alterations have resulted in the progression towards vulvar SCC.

In patient 3, a clonal relationship between vulvar SCC and dVIN was confirmed by several genomic aberrations, including shared copy number alterations and a *TP53* mutation. The most pronounced aberration was a high gain on chromosome 11q23, which encompassed the BIRC and MMP gene clusters as well as the YES-associated protein 1 (YAP1) as a possible candidate gene. This latter gene is known to play a role in the development and progression of multiple cancers and may function as a potential target for cancer treatment [25]. YAP1 expression seems to indicate a poor outcome in several cancer types [26, 27]. Future studies should reveal whether these aberrations are more common in dVIN lesions and are involved in the progression from dVIN towards vulvar SCC. Importantly, the majority of copy number alterations, among which one high-level amplification, were found only in vulvar SCC, and thus may have contributed to the progression towards vulvar SCC. Another alteration preserved from dVIN was a mutation in *TP53*, which was found in low levels in this lesion and became homozygous in a major fraction of tumour cells in the vulvar SCC.

Interestingly, *TP53* mutations were detected in five patients (42%) and, in all cases, were shared between vulvar SCC and dVIN. The mutant allele frequency of these mutations was always lower in the dVIN samples compared to vulvar SCC, indicating that they were present in only a subset of the cells. Whether this low mosaicism was due to heterogeneity of the dVIN lesion or caused by a relatively high load of normal epithelial cells could not be established. In patients 1 and 3, the B-allele frequency plots showed homozygosity of the *TP53* locus in the majority of cells due to acquired uniparental disomy. Our findings illustrate how the detection of acquired uniparental disomy facilitated the identification of *TP53* mutations, but also provide insight into the role of these mutations during tumour progression: *TP53* mutations can find their origin in precursor lesions, as was recently also demonstrated by others [12], and frequently become homozygous in vulvar SCC.

One could question whether the copy number alterations found in the dVIN lesion in patient 3 were actually copy number alterations present in a subset of vulvar SCC cells that were present in the dVIN biopsy. However, although the samples were taken from the same surgical excision specimen, cells for DNA were collected from a different site of the specimen. Furthermore, histological examination of the H&E stained slides (Figure 2) showed a distance of 2 mm with normal epithelium between the dVIN lesion and vulvar SCC lesion. It is important to note that, due to the low copy number intensities in the dVIN sample of patient 3, and the relatively low signal-to-noise ratio, it might be possible that other aberrations detected in the vulvar SCC sample were simply missed in the dVIN sample. Nevertheless, it is unlikely that the high-level amplification at 8q has been missed in dVIN (Figure 3), which indicates that the dVIN and vulvar SCC samples share genomic alterations, but are not identical, thereby making it less likely that vulvar SCC-derived cells were present in the dVIN sample.

Liegl and Regauer [11] suggested that the rare identification of dVIN without vulvar SCC in their patient group raised the question whether dVIN should be considered a true precursor lesion of vulvar SCC or whether it represents an *in situ* carcinoma component adjacent to an invasive vulvar SCC. They suggested that the interpretation of dVIN as a precursor lesion needs to be carefully reconsidered. In general, epithelial disorders are found adjacent to vulvar SCC in 70-80% of patients. However, evidence that some of these disorders are precursors of vulvar SCC is circumstantial [7]. Reason to question dVIN as being the precursor lesion is the low incidence of solitary dVIN compared to HSIL [8], while the majority of vulvar SCCs are not HPV associated. However, this low incidence can also be explained by the difficulty of diagnosing dVIN which might result in an underdiagnosis [9], as well as by the existence of a shorter intra-epithelial phase compared to HSIL [8, 28]. Though there are no recent studies concerning the incidence of dVIN, we experience a higher incidence of solitary dVIN in daily practice because clinicians and pathologists are more aware of the diagnosis.

The number of studies that tried to find genetic similarities between dVIN and vulvar SCC are small and mainly involve a low number of loci investigated [7, 13, 29]. Furthermore, most studies do not differentiate between HSIL and dVIN-related vulvar SCCs. Lin *et al.* [30] compared patterns of loss of heterozygosity between different locations of the tumour of one patient with a non HPV-related vulvar SCC; the tumour itself scored positive for loss of heterozygosity in four of seven loci. Furthermore, one site of dVIN shared its locus with the invasive tumour whereas the other dVIN shared one of two loci with the vulvar SCC. Normal epithelium and stroma showed no abnormalities. These results are suggestive for dVIN being a precursor lesion, though this conclusion is based on a single case and only seven genomic loci. Pinto *et al.* [31] compared 11 identified loci which scored positive for allelic imbalance in greater than 50% of cases from a prior study of vulvar SCC (n=16) [32] to pre-invasive lesions (LS, HSIL, dVIN and hyperplasia). This comparison showed a lower percentage of allelic imbalance in

pre-invasive lesions, though in this comparison no distinction was made between HPV-positive and negative lesions. The advantage of our study is the high number of loci investigated in dVIN associated vulvar SCC, which allows us to compare the whole genome of dVIN and vulvar SCC which provides more detailed information.
A noticeable result is the median age of 59 years of the included patients. Vulvar SCC, especially non HPV-related vulvar SCC, is a disease occurring in elderly patients.
In our study, two out of three dVIN samples analyzed by copy number profiling, did not show copy number aberrations. This observation might be explained by a higher level of contamination with normal cells in these lesions compared to vulvar SCC, or low yield and quality of the isolated DNA, which may have resulted in a decreased sensitivity to detect these aberrations. In addition, however, it is not unlikely that copy number alterations are simply less frequent in these lesions.
In order to provide more evidence for the hypothesis of clonal relationship between LS/dVIN and vulvar SCC, the availability of sufficient material is essential. We experienced that retrieving enough DNA from FFPE-blocks for genome-wide profiling was challenging, and collection of fresh-frozen tissue for future studies is highly recommended. Fortunately, we were able to reveal additional support for our hypothesis by using smMIP-based targeted sequencing of *TP53*, which is frequently mutated in vulvar SCC. This technology is highly suitable for fragmented (FFPE-derived) DNA, and highly sensitive for detecting mutations, even when present in a low percentage of cells. However, in order to obtain more information on the correlation between LS, dVIN and vulvar SCC, whole exome or genome sequencing in a larger number of lesions might be essential, particularly because backtracking of small mutations is less challenging.
In summary, we have investigated the genome-wide aberrations in vulvar SCC and its possible genetic relationship with dVIN and LS, and provided genetic evidence for a clonal relationship between LS, dVIN and vulvar SCC in 42% of the patients, including all patients with *TP53* mutated vulvar SCC. These results will provide a basis for more comprehensive sequencing studies to identify genetic aberrations in precursor lesions that create a risk to the development of vulvar SCC.

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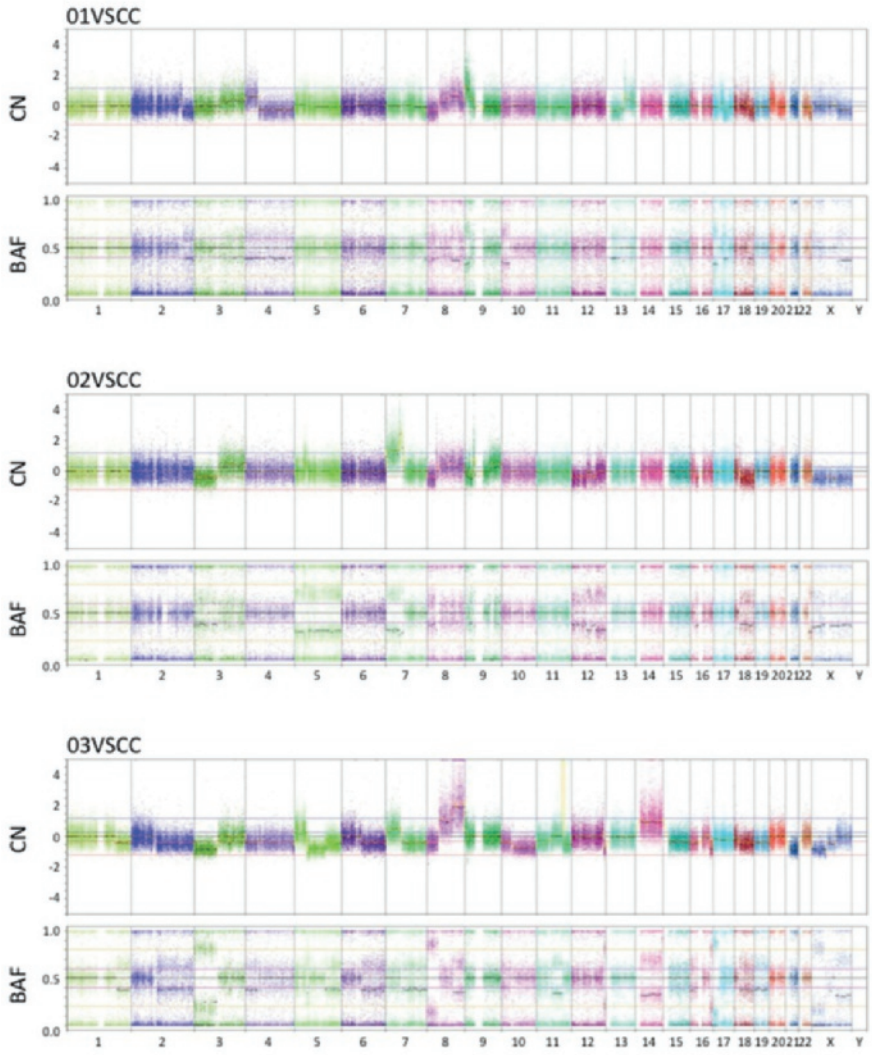
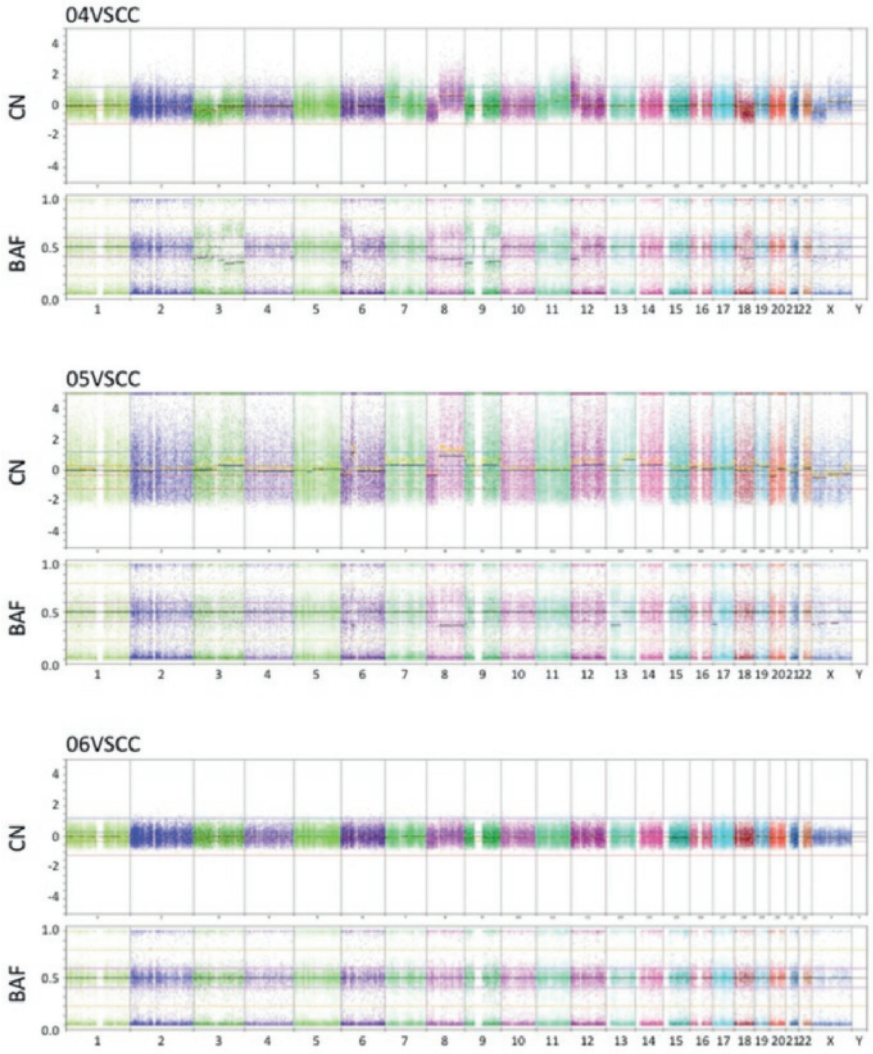
SUPPLEMENTARY DATA



Supplementary Figure 1 Copy number profiles of six vulvar squamous cell carcinoma (VSCC) samples.



Supplementary Figure 1 Continued.

Supplementary Table 1 Small molecule Molecular Inversion probe characteristics.

smMIP name*	smMIP_sequence^		scan_start	scan_stop	orientation
TP53_exon_1.1	acttttgagaagctcaaaacttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtcatcaagttcagtcagg	17	7590688	7590799	+
TP53_exon_1.2	gtgtattttcagctcgggaaaatcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgggctccggggacac	17	7590640	7590751	-
TP53_exon_5.1a	ctagagagagttggcgtctacaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaggggagtactgtagga	17	7578563	7578674	+
TP53_exon_5.1b	ctagggagagttggcgtctacaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaggggagtactgtagga	17	7578563	7578674	+
TP53_exon_5.2	gacagagttgaaagtcagggcacaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTatggccatggcgcgga	17	7578461	7578572	+
TP53_exon_5.3	ggaaggagacagagttgaaagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTctgcttgtagatggccatg	17	7578454	7578565	+
TP53_exon_5.4a	gctgctcaaatagcgatggtgagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTccaagacctgccctgtg	17	7578389	7578500	-
TP53_exon_5.4b	gctgctcagatagcgatggtgagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTccaagacctgccctgtg	17	7578389	7578500	-
TP53_exon_5.5a	gacgcgggtgccgggcggggNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcctgggcaaccagccctgtc	17	7578347	7578458	+
TP53_exon_5.5b	gacgcgggtgccaggcggggNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcctgggcaaccagccctgtc	17	7578347	7578458	+
TP53_exon_5.6	cctcactgattgctcttaggtcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgcagtcacagcacatgac	17	7578309	7578420	-
TP53_exon_5.6b	cctcacggattgctcttaggtcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgcagtcacagcacatgac	17	7578309	7578420	-
TP53_exon_6.1a	accatcgctatttgagcagcgNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtccacacgcaaatttcctt	17	7578257	7578368	+
TP53_exon_6.1b	accatcgctatctgagcagcgNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtccacacgcaaatttcctt	17	7578257	7578368	+
TP53_exon_6.2a	catagtgtgatggtgccctatgagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggttgcccagggtccc	17	7578210	7578321	-
TP53_exon_6.2b	catagtgtggtggtgccctatgagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggttgcccagggtccc	17	7578210	7578321	-
TP53_exon_6.3a	gaggaggggttaagggtggttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcctcctcagcatcttatcc	17	7578151	7578262	-
TP53_exon_6.3b	gagggggggttaagggtggttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcctcctcagcatcttatcc	17	7578151	7578262	-
TP53_exon_6.5	ggtgagcagtagggggcctttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcgtgtggagtatttggatg	17	7578115	7578226	-
TP53_exon_7.1a	catcctcaccatcatcacacNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTccacaggtctccccaaggcg	17	7577532	7577643	-

Supplementary Table 1 Continued.

smMIP name*	smMIP_sequence^	chromosome	scan_start	scan_stop	orientation
TP53_exon_7.1b	catcctcaccatcatcacacNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTccacaggtctccccaaggcn	17	7577532	7577643	-
TP53_exon_7.2	ggaagactccaggtcaggagccaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggcctcatcttgggcct	17	7577511	7577622	-
TP53_exon_7.3a	agataacacaggcccaagatgaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaagtggctcctgacctg	17	7577502	7577613	+
TP53_exon_7.3b	agataacacaggcccaagatgaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaaatggctcctgacctg	17	7577502	7577613	+
TP53_exon_7.4	gttgtagtggatggtggtacagtcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTagaggctggggcacag	17	7577464	7577575	+
TP53_exon_7.5	gtgtgatgatggtgaggatgNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTatgagaggtggatgggtagt	17	7577400	7577511	+
TP53_exon_8.1a	gtttgtgcctgtgctgggagagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggttgggagtagatggag	17	7577119	7577230	-
TP53_exon_8.1b	gtttgtgcctgtcctgggagagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggttgggagtagatggag	17	7577119	7577230	-
TP53_exon_8.2a	caagaggcagtaaggaaatcaggtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgaggctccccttttctt	17	7577068	7577179	+
TP53_exon_8.2b	caagaggcggtaaggaaatcaggtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgaggctccccttttctt	17	7577068	7577179	+
TP53_exon_8.3a	gggagcactaagcgaggtaaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTatcctgagtagtggtaatct	17	7577035	7577146	-
TP53_exon_8.3b	gggagcactaagcgaggtaaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTatcctgagtagtggtgatct	17	7577035	7577146	-
TP53_exon_8.4a	acaggcacaaacatgcacctcaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaccctggtctcctcca	17	7576995	7577106	+
TP53_exon_8.4b	acaggcacaaacacgcacctcaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcaccctggtctcctcca	17	7576995	7577106	+
TP53_exon_8.5a	gagaccaagggtgcagttatgcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgtttgtgcctgtgctgg	17	7576990	7577101	-
TP53_exon_8.5b	gagaccaagggtgcagttatgcNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgtttgtgcctgtcctgg	17	7576990	7577101	-
TP53_exon_9.1	gaagaaaccactggatggagaatNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgtaagcaagcaggacaa	17	7576890	7577001	-
TP53_exon_9.2	gtctcctccaccgcttcttgtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgaagggtgaaatattctc	17	7576873	7576984	+
TP53_exon_9.3	gggacctcttatcaagtggaaaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgcctcagattcactttt	17	7576841	7576952	-
TP53_exon_9.4	Aagaggcaaggaaaggtgata NNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTagtgttagactggaaactt	17	7576821	7576932	+
TP53_exon_9.5	gttttacctgcaattggggcatttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTcctctccccagccaaa	17	7576778	7576889	-
TP53_exon_9.6	ccagtggtttcttctttggctgNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTctgatggcaaatgcccca	17	7576764	7576875	+
TP53_exon_10.1	ggccttggaactcaaggatgNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtcataaagtcaaacaattgt	17	7573990	7574101	-
TP53_exon_10.2	gttcaagttacaattgtttgacttNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTctgggcatccttgagt	17	7573981	7574092	+
TP53_exon_10.3	cagaggaggggagaagtaagtatNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgaggtcactcacctgg	17	7573931	7574042	+

TP53_exon_10.4	attcagctctcggaacatctNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgaatcctatggctttccaac	17	7573880	7573991	+
TP53_exon_10.5	catcctgccttcatggtcaaagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggaactcaaggatgccca	17	7573854	7573965	-
TP53_exon_11.1	gctttgaagggcctaaggctNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTtgaacatgagtttttatgg	17	7572973	7573084	+
TP53_exon_11.2	aggagagatgacatcacatgaNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgtcagtggggaacaagaag	17	7572918	7573029	+
TP53_exon_11.3	ccctcccctgccattttgggtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgccacctgaagtccaaaaa	17	7572879	7572990	-
TP53_90bp_2	GCATGAGACACTTCCAACCCTGGGNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGTTTCCTGACTCAGAG	17	7579876	7579965	+
TP53_90bp_3	ACCCCAACCCCAGCCCCCTNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGTCACTGCCATGGAGGAGCCG	17	7579801	7579900	-
TP53_90bp_4	GCTCGACGCTAGGATCTGACTGCGNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTTTCCCACAGGTCTCTG	17	7579780	7579878	+
TP53_90bp_7	GTCAGTCCCATGGAATTTTCGCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAGGTCCTCAGCCCCCCAG	17	7579642	7579741	+
TP53_90bp_11	GCTCCCAGAATGCCAGAGGCTGCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTTCTTTTCACCCATCTAC	17	7579501	7579592	-
TP53_exon2.1	gatcacctgggcctgcagagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTaaatgtttcctgactcagag	17	7579876	7579987	+
TP53_exon2.1a	ggtcacctgggcctgcagagNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTaaatgtttcctgactcagag	17	7579876	7579987	+
TP53_exon2.3	cccagcccccctagcagagacctNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTggtcactgccatggagga	17	7579793	7579904	-
TP53_exon3.2	ggggttggggtgggggtggtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgtccccagccctccaggtcc	17	7579678	7579789	+
TP53_exon3.2a	GgggttggggtcggggtggtNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTgtccccagccctccaggtcc	17	7579678	7579789	+
TP53_112bp_1	ATCCTAGCGTCGAGCCCCCTCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGGGGGAGGGGGTTCCTTCT	17	7579894	7580005	-
TP53_112bp_2	GGGATCCAGCATGAGACACTTCCANNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTTCCACTCACAGTTTCC	17	7579846	7579957	+
TP53_112bp_3	CCCCAACCCCAGCCCCCTAGCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGCCTTCCGGGTCACTGCCA	17	7579800	7579911	-
TP53_112bp_5	AGGGTTGGGCTGGGGACCTGGNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAAGGGCAGGCCCACCACCC	17	7579691	7579802	-
TP53_112bp_6	GGAATTTTCGCTTCCCACAGGTCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAGGTCCTCAGCCCCCCA	17	7579641	7579752	+
TP53_112bp_7a	GAGGACCTGGTCCTCTGACTGCTCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAAATTCCATGGGACTG	17	7579632	7579743	-
TP53_112bp_7b	GAGGACCTGGTCATCTGACTGCTCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAAATTCCATGGGACTG	17	7579632	7579743	-
TP53_112bp_10a	GGCCCCTGCACCAGCAGCTNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTCCCATCTACAGTCCCCCTTGC	17	7579469	7579580	-
TP53_112bp_10b	GGCCCCTGCACCAGCAGCTNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTCCCATTTACAGTCCCCCTTGC	17	7579469	7579580	-
TP53_112bp_11	GGACAGCATCAAATCATCCATTGCNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGGGGCTGGTGCAGGGG	17	7579437	7579548	+
TP53_112bp_13	GCCGGTGTAGGAGCTGCTGGTGNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTGACCGTGCAAGTCACAGA	17	7579327	7579438	+
TP53_112bp_14	CTGGCTTCCATGAGACTTCAANNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTCCCCCTCCTGGCCCCTGTC	17	7579294	7579405	-
TP53_112bp_15	GTAGGTTTTCTGGGAAGGGACAGNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTAATGCAGGGGGATACGG	17	7579266	7579377	+
TP53_112bp_16	GGCTTCCTGTCAGTGTTTTTTTNNNNNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTCTGGGACAGCCAAGTCTG	17	7579211	7579322	-

(*) a and b reflect smMIPs corresponding to different genotypes of covered SNPs
(^) NNNNNNNN indicates the random 8-nt tag.

Supplementary Table 2 Copy number gains and losses in six VSCC samples.

SampleID	chr	type (*)	start	end	length (Mb)	cytoband
01VSCC	chr02	CN gain	186.058.638	198.479.196	12,42	q32.1 - q33.1
01VSCC	chr02	CN loss	198.950.266	243.199.373	44,25	q33.1 - q37.3
01VSCC	chr03	CN gain	101.634.884	198.022.430	96,39	q12.3 - q29
01VSCC	chr04	CN gain	0	50.400.000	50,40	p16.3 - q11
01VSCC	chr04	CN loss	52.700.771	191.154.276	138,45	q12 - q35.2
01VSCC	chr07	CN loss	151.515.051	159.000.000	7,48	q36.1 - q36.3
01VSCC	chr08	CN loss	0	43.749.726	43,75	p23.3 - p11.1
01VSCC	chr08	**CN gain**	46.950.145	83.121.643	36,17	q11.1 - q21.13
01VSCC	chr08	**CN gain**	83.121.643	146.364.022	60,22	q21.2 - q24.3
01VSCC	chr09	high CN gain	0	21,566,202	21,57	p24.3 - p13.2
01VSCC	chr09	CN loss	21.566.202	22.185.516	0,62	p21.3
01VSCC	chr13	CN loss	19.308.175	69.687.801	50,38	q11 - q21.33
01VSCC	chr13	high CN gain	71.643.837	74.278.611	2,63	q21.33 - q22.1
01VSCC	chr13	CN gain	74.278.611	109.061.226	34,78	q22.1 - q33.3
01VSCC	chr17	CN gain	29.488.572	35.803.400	6,31	q11.2 - q12
01VSCC	chr17	CN loss	41.266.386	57.442.459	16,18	q21.31 - q22
01VSCC	chr18	CN gain	54.127.603	56.207.023	2,08	q21.31 - q21.32
01VSCC	chr18	CN loss	56.207.023	74.381.966	18,17	q21.32 - q23
01VSCC	chr20	CN gain	0	23.222.260	23,22	p13 - p11.21
01VSCC	chr22	CN loss	47.191.586	51.304.566	4,11	q13.31 - q13.33
01VSCC	chrX	CN loss	101.403.500	155.270.560	53,87	q22.1 - q28
02VSCC	chr03	CN loss	0	91.000.000	91,00	p26.3 - q11.1
02VSCC	chr03	CN gain	94.262.831	198.022.430	103,76	q11.2 - q29
02VSCC	chr05	CN gain	37.172.054	50.596.132	13,42	p13.2 - q11.1
02VSCC	chr07	CN gain	0	67.523.814	67,52	p22.3 - q11.22
02VSCC	chr07	high CN gain	51.965.546	59.863.318	7,90	p12.1
02VSCC	chr08	CN loss	0	34.606.454	34,61	p23.3 - p12
02VSCC	chr08	high CN gain	34.606.454	39.007.911	4,40	p12 - p11.22
02VSCC	chr08	CN gain	48.302.428	146.275.000	97,97	q11.21 - q24.3
02VSCC	chr09	CN gain	18.612.221	19.816.014	1,20	p22.1
02VSCC	chr09	CN loss	19.816.014	30.708.447	10,89	p21.3 - p21.1
02VSCC	chr09	high CN gain	32.862.231	37.441.137	4,58	p13.1 - p13.2
02VSCC	chr09	CN gain	93.484.459	141.213.431	47,73	q22.2 - q34.3
02VSCC	chr12	CN loss	0	59.271.881	59,27	p13.33 - q14.1
02VSCC	chr12	CN loss	75.861.975	94.178.228	18,32	q21.2 - q22
02VSCC	chr16	CN loss	20.057.892	32.482.955	12,43	p12.3 - p11.1
02VSCC	chr18	CN loss	21.795.295	78.077.248	56,28	q11.2 - q23
02VSCC	chrX	CN loss	0	155.270.560	155,27	p22.33 - q28
03VSCC	chr01	CN loss	204.214.562	249.250.621	45,04	q32.1 - q43

Supplementary Table 2 Continued.

SampleID	chr	type (*)	start	end	length (Mb)	cytoband
03VSCC	chr01	CN loss	204.214.562	249.250.621	45,04	q32.1 - q43
03VSCC	chr02	CN loss	95.387.135	241.229.436	145,84	q11.2 - q37.3
03VSCC	chr03	CN loss	0	91.000.000	91,00	p26.1 - q11.1
03VSCC	chr03	CN loss	138.144.387	143.070.982	4,93	q22.3 - q24
03VSCC	chr04	CN loss	0	191.154.276	191,15	p16.3 - q35.2
03VSCC	chr05	CN gain	0	52.164.596	52,16	p15.33 - q11.2
03VSCC	chr05	**CN loss**	52.164.596	53.945.232	1,78	q11.2
03VSCC	chr05	**CN loss**	53.945.232	125.355.255	71,41	q11.2 - q23.3
03VSCC	chr05	**CN loss**	125.355.255	174.789.062	49,43	q23.3 - q35.2
03VSCC	chr06	CN loss	79.201.333	158.105.143	78,90	q14.1 - q25.3
03VSCC	chr07	CN gain	22.017.831	55.402.403	33,38	p22.3 - p11.2
03VSCC	chr07	CN loss	61.064.520	151.179.405	90,11	q11.21 - q36.3
03VSCC	chr08	CN loss	0	43.749.726	43,75	p23.3 - p11.1
03VSCC	chr08	CN gain	49.483.834	100.841.341	51,36	q11.21 - q22.2
03VSCC	chr08	high CN gain	100.841.341	146.364.022	45,52	q22.2 - q24.3
03VSCC	chr09	CN gain	0	7.916.102	7,92	p24.3 - p24.1
03VSCC	chr09	CN loss	9.100.000	10.100.000	1,00	p23
03VSCC	chr10	**CN loss**	0	42.365.680	42,37	p15.3 - q11.21
03VSCC	chr10	**CN loss**	42.365.680	134.749.713	92,38	q11.21 - q26.3
03VSCC	chr11	CN loss	0	63.588.519		p15.5 - q13.1
03VSCC	chr11	high CN gain	101.221.509	102.634.411	1,41	q22.1 - q22.2
03VSCC	chr11	CN loss	102.634.411	135.006.516	32,37	q22.3 - q25
03VSCC	chr12	CN loss	127.688.426	133.219.920	5,53	q24.32 - q24.33
03VSCC	chr14	CN gain	9.457.933	107.349.540	97,89	q11.2 - q32.33
03VSCC	chr15	CN loss	20.083.895	102.531.392	82,45	q11.2 - q26.3
03VSCC	chr16	CN loss	0	35.166.094	35,17	p13.3 - p11.2
03VSCC	chr16	CN loss	78.702.625	90.354.753	11,65	q23.3 - q24.1
03VSCC	chr17	CN loss	0	81.021.937	81,02	p13.3 - q25.3
03VSCC	chr18	CN loss	18.535.950	78.077.248	59,54	q11.2 - q23
03VSCC	chr19	CN loss	0	59.095.126	59,10	p13.3 - q13.43
03VSCC	chr21	CN loss	14.350.083	48.129.895	33,78	q11.2 - q22.3
03VSCC	chrX	CN loss	2.699.968	58.545.809	55,85	p22.33 - p11.1
03VSCC	chrX	CN loss	61.830.816	92.615.392	30,78	q12 - q21.31
04VSCC	chr03	CN loss	0	95.977.995	95,98	p26.3 - q11.2
04VSCC	chr07	CN gain	0	57.877.934	57,88	p22.3 - p11.2
04VSCC	chr08	CN loss	0	43.749.726	43,75	p23.2 - p11.1
04VSCC	chr08	CN gain	46.950.145	146.364.022	99,41	q11.1 - q24.3
04VSCC	chr09	CN loss	8.431.596	9.932.940	1,50	p24.1 - p23
04VSCC	chr11	CN gain	57.135.371	135.006.516	77,87	q12.1 - q25

Supplementary Table 2 Continued.

SampleID	chr	type (*)	start	end	length (Mb)	cytoband
04VSCC	chr11	CN gain	57.135.371	135.006.516	77,87	q12.1 - q25
04VSCC	chr12	CN gain	0	34.778.715	34,78	p13.33 - p11.1
04VSCC	chr18	CN loss	32.054.846	78.077.248	46,02	q12.1 - q23
04VSCC	chrX	CN loss	2.699.968	58.545.809	55,85	p22.33 - p11.1
05VSCC	chr03	CN gain	93.595.962	198.022.430	104,43	q11.1 - q29
05VSCC	chr06	CN loss	0	39.215.986	39,22	p25.3 - p21.2
05VSCC	chr06	CN gain	39.215.986	58.742.393	19,53	p21.2 - p11.1
05VSCC	chr07	CN gain	0	159.138.663	159,14	p22.3 - q36.3
05VSCC	chr08	CN loss	0	43.749.726	43,75	p23.3 - p11.1
05VSCC	chr08	CN gain	46.950.145	146.364.022	99,41	q11.1 - q24.3
05VSCC	chr09	CN gain	0	141.213.431	141,21	p24.3 - q34.3
05VSCC	chr12	CN gain	0	133.851.895	133,85	p13.33 - q24.33
05VSCC	chr13	CN gain	72.971.234	115.169.878	42,20	q21.33 - q34
05VSCC	chr14	CN gain	19.605.413	107.349.540	87,74	q11.2 - q32.33
05VSCC	chr20	CN loss	0	26.224.651	26,22	p13 - p11.1
05VSCC	chrX	**CN loss**	2.699.968	58.545.809	55,85	p22.33 - p11.1
05VSCC	chrX	**CN loss**	61.830.816	155.270.560	93,44	q11.1 - q28
06VSCC	chr08	CN loss	0	43.749.726	43,75	p23.2 - p11.21

(*) copy number types indicated in bold represent adjacent gains or losses with different intensities

Supplementary Table 3 Recurrently affected copy number regions.

chr	type (*)	Recurrence	start	end	length (Mb)	Cyto band	Affected samples (*)
chr03	CN gain	3	101.634.884	198.022.430	96,39	q12.3 - q29	01VSCC, 02VSCC, 05VSCC
chr05	CN gain	2	37.172.054	50.596.132	13,42	p13.2 - q11.1	02VSCC, 03VSCC
chr07	CN gain	4	51.965.546	55.402.403	3,44	p22.3 - p11.2	**02VSCC**, 03VSCC, 04VSCC, 05VSCC
chr08	CN gain	5	49.483.834	100.841.341	51,36	q11.21 - q22.2	01VSCC, 02VSCC, 03VSCC, 04VSCC, 05VSCC
chr09	CN gain	3	0	7.916.102	7,92	p24.3 - p24.1	01VSCC, 03VSCC, 05VSCC
chr09	CN gain	3	32.862.231	37.441.137	4,58	p13.1 - p13.2	01VSCC, **02VSCC**, 05VSCC
chr09	CN gain	3	18.612.221	19.816.014	1,20	p22.1	01VSCC, 02VSCC, 05VSCC
chr11	CN gain	2	101.221.509	102.634.411	1,41	q22.1 - q22.2	**03VSCC**, 04VSCC
chr12	CN gain	2	0	34.778.715	34,78	p13.33 - p11.1	04VSCC, 05VSCC
chr13	CN gain	2	71.643.837	74.278.611	2,63	q21.33 - q22.1	**01VSCC**, 05VSCC
chr13	CN gain	2	74.278.611	109.061.226	34,78	q22.1 - q33.3	01VSCC, 05VSCC
chr14	CN gain	2	19.605.413	107.349.540	87,74	q11.2 - q32.33	03VSCC, 05VSCC
chr02	CN loss	2	95.387.135	241.229.436	145,84	q11.2 - q37.3	01VSCC, 03VSCC
chr02	CN loss	2	198.950.266	243.199.373	44,25	q33.1 - q37.3	01VSCC, 03VSCC
chr03	CN loss	3	0	91.000.000	91,00	p26.3 - q11.1	02VSCC, 03VSCC, 04VSCC
chr04	CN loss	2	52.700.771	191.154.276	138,45	q12 - q35.2	01VSCC, 03VSCC
chr08	CN loss	6	0	34.606.454	34,61	p23.3 - p12	01VSCC, 02VSCC, 03VSCC, 04VSCC, 05VSCC, 06VSCC
chr09	CN loss	2	21.566.202	22.185.516	0,62	p21.3	01VSCC, 02VSCC
chr16	CN loss	2	20.057.892	32.482.955	12,43	p12.3 - p11.1	02VSCC, 03VSCC
chr17	CN loss	2	41.266.386	57.442.459	16,18	q21.31 - q22	01VSCC, 03VSCC
chr18	CN loss	4	56.207.023	74.381.966	18,17	q21.32 - q23	01VSCC, 02VSCC, 03VSCC, 04VSCC
chrX	CN loss	3	2.699.968	58.545.809	55,85	p22.33 - p11.1	03VSCC, 04VSCC, 05VSCC
chrX	CN loss	3	61.830.816	92.615.392	30,78	q12 - q21.31	01VSCC, 03VSCC, 05VSCC

(*) Samples indicated in bold and underlined carry high-level amplifications at these positions

Supplementary Table 4 Regions of copy-neutral loss-of-heterozygosity (LOH).

SampleID	chr	type	start	end	length (Mb)	cytoband
04VSCC	chro3	LOH	95.977.995	191.154.276	95,18	q11.2 - q29
02VSCC	chro5	LOH	0	174.789.062	174,79	p15.33 - q35.2
04VSCC	chro6	LOH	0	42.131.807	42,13	p25.3 - p21.1
04VSCC	chro9	LOH	0	33.234.269	33,23	p24.1 - p21.1
04VSCC	chro9	LOH	80.384.220	141.213.431	60,83	q21.2 - q34.3
01VSCC	chr10	LOH	0	33.544.391	33,54	p15.3 - p11.22
03VSCC	chr11	LOH	63.588.519	101.289.628	37,70	q13.1 - q22.2
02VSCC	chr12	LOH	94.178.228	133.851.895	39,67	q22 - q24.33
05VSCC	chr13	LOH	19.308.175	58.905.048	39,60	q12.11 - q21.1
01VSCC	chr17	LOH	0	17.974.077	17,97	p13.3 - p11.2
03VSCC	chr17	LOH	0	19.662.176	19,66	p13.3 - p11.2
05VSCC	chr17	LOH	0	16.807.475	16,81	p13.3 - p11.2

Measuring the depth of invasion in vulvar squamous cell carcinoma: interobserver agreement and pitfalls

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ABSTRACT

Aims The depth of invasion is an important prognostic factor for patients with vulvar squamous cell carcinoma (SCC). The threshold of 1 mm distinguishes between FIGO stage IA and ≥ IB disease and guides the need for groin surgery. Therefore, high interobserver agreement is crucial. The conventional and the alternative method are described to measure the depth of invasion. The aims of this study were to assess interobserver agreement for classifying the depth of invasion using both methods and to identify pitfalls.

Methods Fifty slides of vulvar SCC with a depth of invasion approximately 1 mm were selected, digitally scanned and independently assessed by ten pathologists working in a referral or oncology center, and four pathologists in training. The depth of invasion was measured using both the conventional and alternative method in each slide and categorized into ≤ 1 and › 1 mm. The percentage of agreement and Light's kappa for multi-rater agreement were calculated and 95% confidence intervals were calculated by bootstrapping (1000 runs).

Results The agreement using the conventional method was moderate (kappa = 0.57 (95% confidence interval 0.45-0.68)). The percentage of agreement among the participating pathologists using the conventional method was 85.0 versus 89.4% using the alternative method. Six pitfalls were identified; disagreement concerning which invasive nest is deepest, recognition of invasive growth and where it starts, curved surface, carcinoma situated on the edge of the tissue block, ulceration and different measurement methods.

Conclusions Pathologists reached only moderate agreement in determining the depth of invasion in vulvar SCC, without a notable difference between the two measurement methods.

INTRODUCTION

It is generally accepted that tumor thickness and/or depth of invasion (DOI) is a reliable parameter for predicting the likelihood of regional lymph node involvement and survival in many malignancies, such as cervical, head and neck and colorectal cancers [1-3]. The DOI is also an important prognostic factor in patients with vulvar squamous cell carcinoma (SCC) and determines the need for groin surgery. Early stage vulvar SCC is treated by radical local excision of the tumor, with or without inguinofemoral lymph node staging, depending on the DOI [4]. In patients with a microinvasive carcinoma (DOI ≤ 1 mm, FIGO stage IA), the risk of inguinofemoral lymph node metastases is negligible and lymph node staging can be safely omitted [5, 6]. In patients with macroinvasive disease (DOI > 1 mm, FIGO stage ≥ IB) a sentinel node procedure and/or an inguinofemoral lymphadenectomy is indicated. Inguinofemoral lymphadenectomy is associated with significant morbidity. This morbidity encompasses short-term morbidity including wound infection, formation of lymphoceles and/or wound breakdown in up to 85% of the patients and long-term morbidity including lymphedema, cellulitis and erysipelas in up to 64% of the patients [7-9]. Because of the far reaching consequences of inguinofemoral lymphadenectomy, classification of the DOI with a threshold of 1 mm is crucial and a high interobserver agreement is important.

Wilkinson *et al.* have described a number of methods for measuring the DOI in vulvar SCC [10]. The International Federation of Gynecology and Obstetrics (FIGO) recommend to '*measure from the epithelial-stromal junction of the most superficial adjacent dermal papillae to the deepest point of invasion*', as shown in Figure 1, method A [11]. In many carcinomas such as cervical cancer, the depth of invasion is measured from the nearest dysplastic crypt or surface epithelium [1]; because logically tumor cells will originate from the nearest rete ridges instead of the most superficial dysplastic epithelium. In vulvar cancer, this measurement method (*measurement from the most adjacent dysplastic abnormal rete ridge to the deepest point of invasion*) is analogous to the method used in cervical cancer (see Figure 1; method B).

This alternative measurement method has been studied by Van den Einden *et al.* [12]; they performed a retrospective study comparing the DOI measured by both the conventional and alternative method in a series of vulvar carcinoma, and concluded that this alternative method may provide a better reflection of the prognosis. With a cut-off of 1 mm for both methods, the alternative method resulted in downstaging of the FIGO stage in 9% of the patients (14 of 148). In 13 patients (19%) with FIGO stage IB disease, the carcinoma was downstaged to stage IA, in which no groin surgery is indicated. In none of patients was there evidence of lymph node metastasis. However, in one downstaged patient from FIGO stage IIIA isolated tumor cells were present in the lymph node removed by the sentinel node technique.

The question was raised of whether there is a difference in the interobserver agreement when pathologists use the method recommended by FIGO or an alternative method as described above. We aimed to assess the interobserver agreement between pathologists using two different measurement methods and to identify pitfalls in the assessment of the DOI.

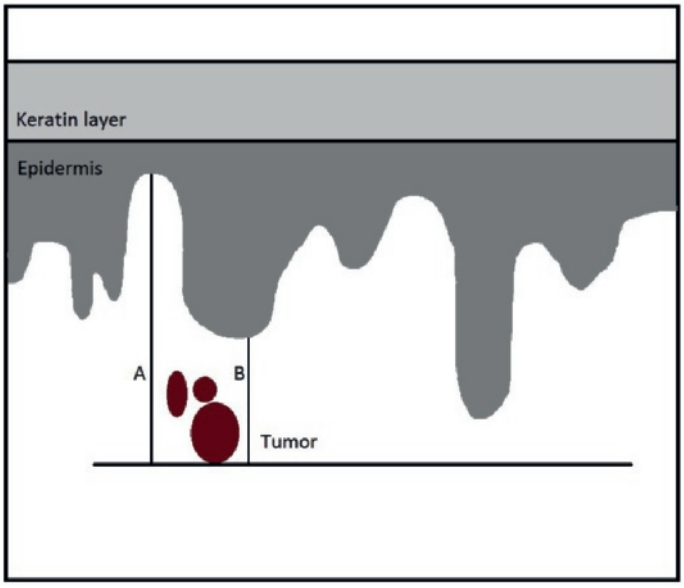

Figure 1 Measurement methods for the depth of invasion in vulvar squamous cell carcinoma.

Method A: conventional method; measurement(s) from the epithelial-stromal junction of the most superficial adjacent dermal papillae to the deepest point of invasion
Method B: Alternative method; measurement from the most adjacent dysplastic abnormal rete ridge to the deepest point of invasion

METHODS

Slides from biopsies and/or surgical resection specimens of patients treated for vulvar squamous cell carcinoma at the Radboud university medical center between 2000 and 2017 were retrieved. An expert gynaecologic pathologist (JB) reviewed and selected slides for inclusion; both diagnostically challenging and straightforward slides were selected, representing daily practice. In all slides there was a DOI of approximately 1 mm: approximately half the slides showed a DOI ≤ 1.0 mm, and half

› 1.0 mm at the initial histopathological examination measured by the conventional method. The area of invasion was circled on the slide and all slides were anonymized. All slides were assessed independently by all participants; working in either a gynecologic oncology center or a referring hospital. The expert pathologist (JB) who selected the slides for inclusion did not participate in the study. For each individual slide, participants measured the DOI using both the conventional and the alternative methods using a digital ruler. The digital ruler measures the distance between two locations and a straight line was displayed. Each measurement was reported in mm, with an accuracy of one decimal point in an online questionnaire using Castor EDC [13]. After assessing the slides, the participants recorded how certain they were about each measurement and noted any difficulties and/or comments. Furthermore, the participants were asked what method they used in daily practice and how many years of experience they had. We based our sample size on a previous study which evaluated the interobserver agreement when assessing the DOI of vulvar SCC [14]. We estimated that the kappa for interobserver agreement for the DOI ≤ 1 mm versus › 1 mm using the conventional measuring method would be approximately 0.70 (standard deviation 0.10). With ten participating pathologists, a power of 80%, an alpha of 5%, a two-sided 95% confidence interval (CI) of maximal 0.10, 50 slides were required for pathological assessment [15]. In addition, we included four pathology residents to assess all 50 slides in order to identify differences in the interobserver agreement between residents and pathologists. The slides were digitally scanned (Pannoramic P250 Flash II, 3D histech) and distributed to the participants using tEPIS (Trait Enhanced Pathology Image Sharing-system), a digital pathology platform. The participants were not informed about the original diagnosis, did not receive any clinical information and were not aware of the measurements made by other participants. The participants received Figure 1 as instruction on how to perform both measurement methods. The conventional method was defined as; '*measurement(s) from the epithelial-stromal junction of the most superficial adjacent dermal papillae to the deepest point of invasion*'. The alternative method was defined as '*measurement from the most adjacent dysplastic abnormal rete ridge to the deepest point of invasion*'.

The annotations made on the slides by each participant were visible to the researcher and were reviewed by the expert gynecologic pathologist (JB); this gave the pathologist insight into where exactly the measurement had been made and allowed review of the discordant slides and to analysis of the reasons for discrepancies to identify pitfalls.

Statistical analysis

For purposes of analysis, the DOI measurements were dichotomized into two categories, DOI ≤ 1.0 mm and › 1.0 mm, as this categorization is clinically relevant. The percentage of interobserver agreement was calculated separately for the conventional and the alternative methods for diagnostically challenging and straightforward slides, and for

pathologists working in an gynecologic oncology center or referring hospital. Light's kappa for multi-rater agreement was calculated for the conventional method and 95% CIs were calculated by bootstrapping (1000 runs). Kappa values were interpreted as slight (‹0.21), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) or almost perfect (0.81-0.99) interobserver agreement [16].
A slide was arbitrarily defined as discordant if there was agreement on the DOI, classified as microinvasive (DOI ≤1 mm, FIGO stage IA) or macroinvasive ((DOI › 1 mm, FIGO stage ≥ IB) among fewer than seven of ten pathologists (≤ 60%), using either the conventional or the alternative method. The statistical software R was used for statistical analysis (version 3.3.2), with the 'irr' package.

Ethics statement

Anonymised residual tissue was used, which was retrieved during regular treatment. According to Dutch law, no specific patient approval is necessary for the use of this material. This study was approved by the local ethical committee (number 2016-2728) and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies (htpp://federa.org).

RESULTS

Of the 50 slides selected, 24 (48%) were diagnosed as microinvasive (DOI ≤ 1 mm, FIGO stage IA) and 26 (52%) macroinvasive (DOI ›1 mm, FIGO stage ≥IB) at initial histopathological examination. Ten pathologists assessed all 50 slides; there was a median of 10 years' experience as a pathologist (range 0.5-35 years). Five pathologists worked in an gynecologic oncology center and five in a referring hospital all within Europe; eight in the Netherlands, one in Belgium and one in Spain. Additionally, four residents, all working in an oncology center in the Netherlands, assessed all study slides. According to the participating pathologists, microinvasive growth (DOI ≤ 1 mm, FIGO stage IA) was present in 32-72% and macroinvasive growth (DOI › 1 mm, FIGO stage ≥ IB) in 22-66% of the study slides using the conventional method, see Table 1. The alternative method resulted in downgrading from macroinvasive growth (DOI › 1 mm or FIGO stage ≥ IB) into microinvasive growth (DOI ≤ 1 mm, FIGO stage IA) in 52-80% of the slides assessed as macroinvasive (DOI › 1 mm or FIGO stage ≥ IB) growth using the conventional method, see Table 1.

The agreement among pathologists in the assessment of the DOI was moderate (kappa 0.57 (95%CI 0.45-0.68)) using the conventional method. The percentage of agreement among the participating pathologists using the conventional method was 85.0% versus 89.4% using the alternative method. As shown in Table 2, in diagnostically

Table 1 Measurements of pathologists using the conventional method in relation to the original diagnosis and the number of slides downgraded from macroinvasive (DOI › 1 mm, FIGO stage ≥IB) to microinvasive (DOI ≤ 1 mm, FIGO stage IA) by using the alternative method to measure the depth of invasion

Pathologist	Microinvasive N (%)	Macroinvasive N(%)	Not assessed N (%)	Downgraded N (%)
Original diagnosis	24 (48)	26 (52)		
1	19 (38)	30 (60)	1 (2)	21/30 (70)
2	21 (42)	29 (58)	0	16/29 (55)
3	19 (38)	31(62)	0	20/31 (65)
4	26 (51)	23 (47)	1 (2)	12/23 (52)
5	20 (40)	30 (60)	0	24/30 (80)
6	17 (34)	33 (66)	0	19/33 (59)
7	36 (72)	11 (22)	3 (6)	6/11 (55)
8	20 (40)	30 (60)	0	23/30 (77)
9	16 (32)	32 (64)	2 (4)	22/32 (69)
10	25 (50)	24 (48)	1 (2)	13/24 (54)

challenging slides the agreement was higher using the alternative compared to the conventional method.

Pathologists working in an oncology center reached higher agreement than those from the referring centers for both the conventional method (88.0% versus 83.2%, respectively) and the alternative method (91.6% versus 88.8%, respectively), see Table 2. Using the conventional method, full agreement by the pathologists was obtained in 34% (17 of 50) of the slides and five slides (five of 50, 10%) were considered as discordant; in one slide agreement was 40%, in two 50% and in two 60%. For measurements made by the alternative method full agreement by the pathologists was obtained in 54% (27 of 50) of the slides and four slides were considered as discordant; agreement was 50% in one and 60% in the others. One slide was included in both groups.

As shown in Table 2, agreement between residents was 93.5% using the conventional method and 89.5% using the alternative method. There was full agreement between all four residents in 84% (42 of 50) and 72% (36 of 50) of the slides, respectively. There were more discordant slides using the alternative method (10%) compared to the conventional method (6%).

Table 2 Agreement among pathologists (N=10) and pathologists in training (N=4) in assessing the depth of invasion

Subgroups	Conventional method	Alternative method
Pathologists		
Overall agreement	85.0%	89.4%
Slides		
Straightforward (N=30)	86.3%	91.3%
Diagnostically challenging (N=20)	83.0%	86.5%
Type of center		
Oncology (N=5)	88.0%	91.6%
Referring (N=5)	83.2%	88.8%
Slides with full agreement	34.0%	54.0%
Discordant slides (agreement ≤60%)	10.0%	8.0%
Residents		
Overall	93.5%	89.5%
Slides		
Straightforward (N=30)	95.8%	90.8%
Diagnostic challenging (N=20)	90.0%	87.5%
Slides with full agreement	84.0%	72.0%
Discordant slides (agreement ≤60%)	6.0%	10.0%

Of the ten participating pathologists, seven (70%) used the conventional method and two (20%) the alternative method to measure the DOI in daily practice. One (10%) pathologist used a combination of the two methods, using the alternative method in tumors with early stromal invasions or microinvasion.
Three of the four (75%) residents used the conventional method in daily practice. One (25%) used a combination of the conventional and alternative methods (the alternative method in certain cases with microinvasion).

All pathologists scored ease of use on a scale from 1 to 5 (1= very difficult, 5= very easy). The ease of use for the conventional method was scored as a median 4 of 5 points (range 1-5), and the alternative method as a median 4 of 5 points (range 1-4). Half the pathologists (five of 10) scored both methods equally, three pathologists gave the conventional method a higher score and two scored the alternative method more highly.

All pathologists scored how sure they were about their measurement on a scale from 1 to 5 (1 = not sure at all, 5 = very sure). Eight pathologists were equally sure about their measurement using both methods, one pathologist was more sure about the measurements using the conventional method and one using the alternative method. Overall score was median 3 of 5 points for the conventional method versus median 3 for the alternative method.

Discordant slides were reviewed by the expert gynecologic pathologist (JB) to analyze the reasons for discrepancies. This resulted in the identification of six pitfalls in the assessment of the DOI: (1) disagreement on which invasive nest is deepest (Figure 2A-C), (2) the recognition of whether or not there is in fact invasive growth and where it starts

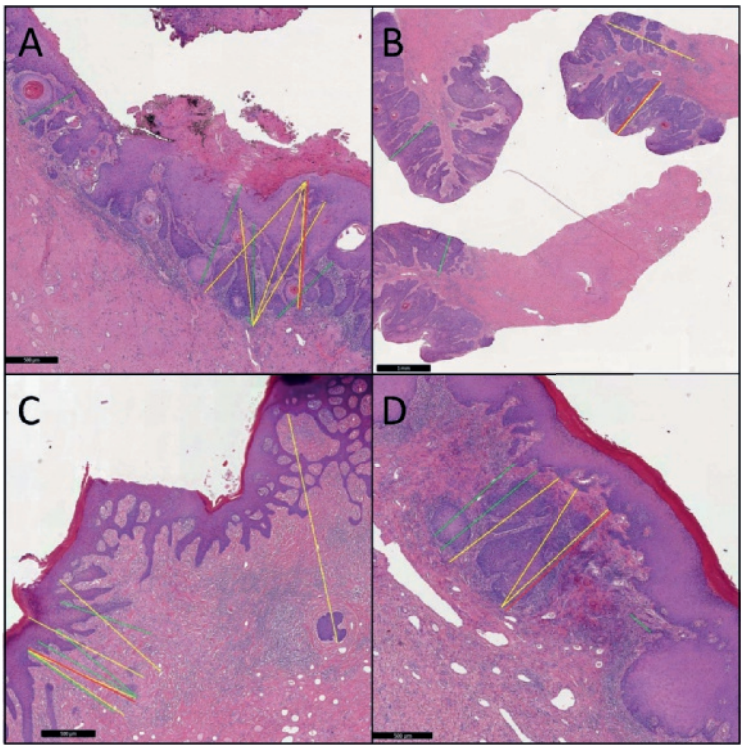

Figure 2 Depth of invasion measured by different pathologists in discordant slides.

Footnote: The yellow and green lines are a measurement of at least one pathologist; macroinvasive (DOI >1 mm, FIGO stage ≥ IB) measurements are displayed in yellow, microinvasive (DOI ≤1 mm, FIGO stage IA) measurements are displayed in green. In red, the recommended measurement is displayed.
In A, B, C, G and H, the conventional method was used and in D, E, F and I the alternative method was used to measure the depth of invasion. In Figure J, both the conventional and alternative method are displayed.

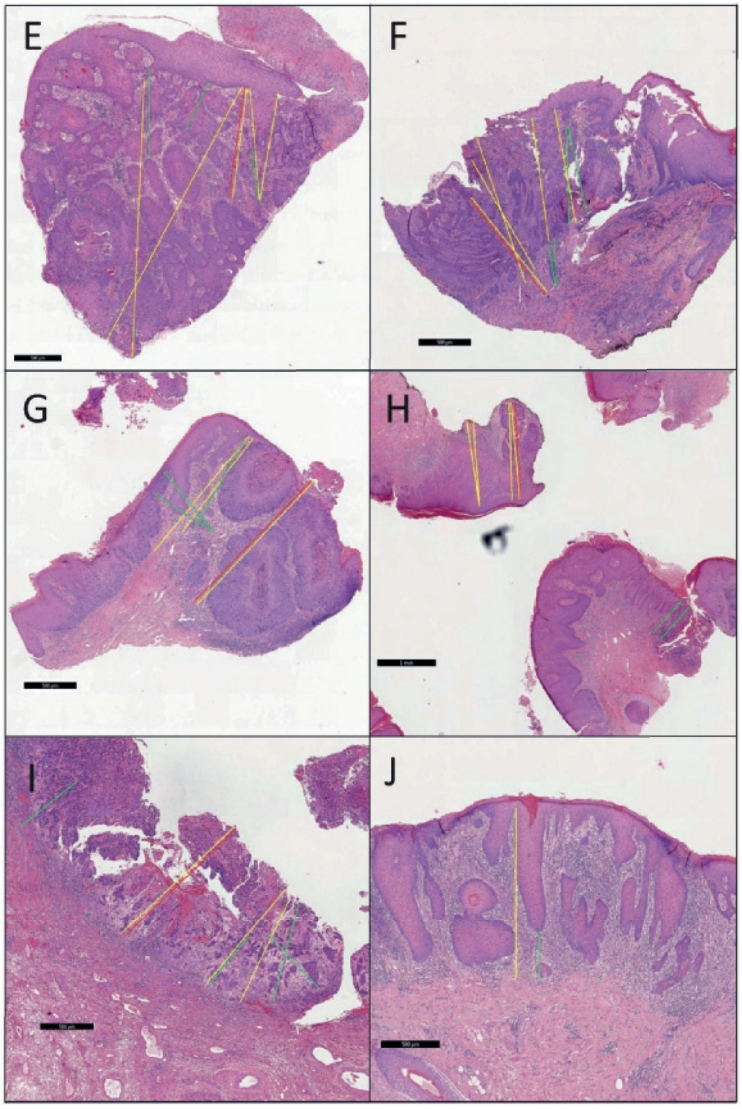

Figure 2 Continued.

(Figure 2B-F), (3) a curved surface (Figure 2G), (4) a carcinoma situated on the edge of the tissue block (Figure 2H), (5) ulceration (Figure 2I) and (6) different methods are used to measure the DOI (Figure 2J). Subsequently, the recommended measurements by the expert gynecologic pathologist are displayed in red in Figure 2.

Discussion

A threshold of 1 mm in the DOI distinguishes between micro- and macroinvasive growth (FIGO stages IA and ≥ IB) and guides the need for groin surgery. Reproducibility of this measurement is important and clinically relevant. However, among pathologists there is only moderate agreement (kappa 0.57 (95% CI 0.45-0.68)) in the assessment of the DOI using the conventional method as recommended by the FIGO.

The results of our study showing moderate (kappa 0.51) interobserver agreement between 11 pathologists for classifying the DOI are in line with another study [14]. This study encouraged the participants to use the conventional method to measure the DOI, but only one of 11 participants was able to use this method in all 45 cases. This underlines the difficulty of measuring the DOI in vulvar SCC and the variation in methods of measurement used by pathologists. Our study confirms the result that measuring invasion depth is indeed difficult in vulvar SCC. Additionally, we offer a unique insight into the difficulties of measuring the depth of invasion by the use of digital pathology. In-depth analyses of all discrepant slides identified six pitfalls. Based on the depicted pitfalls, we formulated recommendations for assessing the DOI in vulvar SCC, as displayed in Table 3. Besides these recommendations, further improvement can be achieved by education, for which the discordant slides and the formulated pitfalls and recommendations of our study are an excellent base.

We showed that pathologists reach similar agreement for the classification of the DOI into a micro- or macroinvasive carcinoma (FIGO stages IA and ≥ IB) using the conventional and alternative methods. In contrast, pathologists in training reached higher agreement using the conventional method. This might be explained by recent training concerning the conventional method and therefore more homogeneity.

The strengths of our study are the international participation in the study, the participation of pathologists working in both referring and oncology centers and the inclusion of slides representing daily clinical practice, i.e. both straightforward and diagnostically challenging slides. In addition, for several reasons, a unique strength is the use of digital pathology for several reasons. First, digital pathology uses a digital ruler and makes it easier for the pathologist to perform the measurements. Secondly, digital pathology makes it easy to share pictures of the slides between different pathologists for revision and more importantly the point of deepest invasion and the measurement made by the pathologist are visible for other consulted pathologists in case of doubt. Thirdly, digital images enabled the researchers to perform in depth analyses of the measurements made and allowed analyses of discrepant slides and identification of pitfalls.

A possible limitation of our study is the statistical method used. We dichotomized the measurement of the individual pathologists into ≤ 1 and › 1 mm, as these outcomes are clinically relevant. This may have introduced imprecision, as in some slides the DOI

was very close to 1 mm. We were not able to compare the kappa for both methods, because the kappa is dependent on the distribution of the micro- and macroinvasive slides between the groups (DOI ≤1 mm and >1 mm, FIGO stage IA and ≥IB). This distribution was different for both methods, as the alternative method is more likely to result in more carcinomas being classified as microinvasive (DOI ≤1 mm, FIGO stage IA) compared to the conventional method. Another limitation is the selection of slides with a DOI of approximately 1 mm instead of consecutive series. This might have resulted in an underestimation of the interobserver agreement and an overestimation of the percentage of slides downgraded from stage ≥ IB measured by the conservative method to FIGO stage IA measured by the alternative method.
In conclusion, this study showed only moderate agreement between pathologists classifying the DOI into micro- and macroinvasive vulvar SCC (FIGO stages IA and ≥ IB)

Table 3 Recommendations based on the pitfalls in the assessment of the depth of invasion in vulvar squamous cell carcinoma.

Pitfalls	Recommendations	Examples, see Figure 2
1. Recognition which invasive nest is deepest	• In tumors ≤1 cm; totally embed the carcinoma • If still uncertain, cut at least two deeper levels on the block	A-C
2. Recognition whether or not there is in fact invasive growth and where it starts	• See recommendations of pitfall 1. • Tumors >1 cm; enclose one tissue block for every 0.5 cm of the carcinoma	B- F
3. Curved surface with two or more possible locations of the surface	• Measure from the surface resulting in the least favorable depth of invasion	G
4. Carcinoma situated on the edge of the tissue block	• Locate the carcinoma in the middle of the block if possible	H
5. Ulceration	• Sample the carcinoma without ulceration. If not possible, measure from the floor of the tumor ulcer	I
6. Different measurement methods are used	• Use the conventional method. Do not routinely use the alternative method until validated. But if used, state the method of measurement used in the pathology report	J
In case of doubt if micro- or macroinvasive growth (FIGO stage IA or ≥IB) is present in the carcinoma after following the above recommendations, we advise consultation with an expert gynecopathologist.		

using the conventional measurement method recommended by the FIGO, and similar agreement using the alternative method. This study showed that the alternative method is suitable for pathologists to measure and classify the DOI in vulvar SCC. However, before implementing this method in daily clinical practice, future research should be performed to determine if the alternative method leads to a better reflection of the prognosis, and of whether a new threshold needs to be defined to reflect biological tumor behavior.

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4

Prognostic factors for local recurrence of squamous cell carcinoma of the vulva: a systematic review

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ABSTRACT

Background In patients treated for early-stage vulvar squamous cell carcinoma local recurrence is reported in up to 40% after ten years. Knowledge on prognostic factors related to local recurrences should be helpful to select high risk patients and/or to develop strategies to prevent local recurrences.

Objective This systematic review aims to evaluate the current knowledge on the incidence of local recurrences in vulvar carcinoma related to clinicopathologic and cell biologic variables.

Data sources Relevant studies were identified by an extensive online electronic search in July 2017.

Study eligibility criteria Studies reporting prognostic factors specific for local recurrences of vulvar carcinoma were included.

Study appraisal and synthesis methods Two review authors independently performed data selection, extraction and assessment of study quality. The risk difference was calculated for each prognostic factor when described in two or more studies.

Results Twenty-two studies were included; most of all were retrospective and mainly reported pathologic prognostic factors. Our review indicates an estimated annual local recurrence rate of 4% without plateauing. The prognostic relevance for local recurrence of vulvar carcinoma of all analyzed variables remains equivocal, including pathologic tumor free margin distance ‹ 8 mm, presence of lichen sclerosus, groin lymph node metastases and a variety of primary tumor characteristics (grade of differentiation, tumor size, tumor focality, depth of invasion, lymphovascular space invasion, tumor localization and presence of human papillomavirus).

Conclusions Current quality of data on prognostic factors for local recurrences in vulvar carcinoma patients does not allow evidence-based clinical decision making. Further research on prognostic factors, applying state of the art methodology is needed to identify high-risk patients and to develop alternative primary and secondary prevention strategies.

INTRODUCTION

Vulvar cancer accounts for 5% of all gynecologic cancers with an incidence of 2.5 per 100,000 women [1]. It mostly affects elderly women, with more than half of the patients above the age of 70 years at time of diagnosis. The most common histological type of vulvar cancer is squamous cell carcinoma [2]. There are two different preneoplastic lesions known for vulvar carcinoma; differentiated vulvar intraepithelial neoplasia (dVIN) and high-grade squamous intraepithelial lesion (HSIL) [3]. The dVIN pathway is associated with lichen sclerosus, the second pathway is related to HSIL which is caused by infection with human papillomavirus (HPV) and associated with immunosuppressive state and smoking [4]. Over the past few decades, the incidence of vulvar carcinoma has increased slowly, but steadily [1].

Standard treatment for early-stage vulvar carcinoma entails wide local excision of the primary tumor and, if the tumor is macroinvasive (depth of invasion > 1mm), either a sentinel node (SLN) procedure or an elective inguinofemoral lymphadenectomy, depending on tumor size, focality or the presence of suspected metastatic groin lymph nodes [5]. Despite radical treatment, local recurrences on the vulva are reported in up to 40% of the patients [6]. Of these patients 43-72% will develop a second local recurrence and subsequently 57% will have a third or even more local recurrences. It has been shown that disease-specific survival decreases from 90% to 69% in patients after a local recurrence, as was observed both in SLN-positive as well as in SLN-negative patients [6].

In patients with a local recurrence, first choice of treatment is wide local excision of the tumor. An inguinofemoral lymphadenectomy is indicated if a macro-invasive recurrent tumor is present and no inguinofemoral lymphadenectomy was performed previously. As a consequence, patients who previously had a negative SLN will now suffer from significant short and long term morbidity associated with inguinofemoral lymphadenectomy [5]. For these reasons, knowledge on prognostic factors related to local recurrences is of utmost importance. Both clinicopathologic as well as cell biologic markers may be of prognostic value for local recurrences of vulvar carcinoma [7, 8].

One of the most debated prognostic factors is the minimal pathologic tumor free margin distance. Worldwide a pathologic tumor free margin of ≥ 8 mm has been advocated as safe [9, 10]. However, the question whether a margin distance < 8mm does really increase the number of patients suffering from a local recurrence has not been answered unequivocally yet. Furthermore, it is unclear why some patients with lichen sclerosus and dVIN develop a primary vulvar squamous cell carcinoma and/or multiple local recurrences, whereas other patients do not.

The aim of this systematic review was to summarize the current knowledge on the incidence of local recurrences in patients diagnosed with vulvar squamous cell carcinoma related to clinicopathologic and cell biologic variables.

METHODS

Information sources and search strategy

This review was conducted according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines, and in accordance with the principles outlined in the Cochrane Handbook for Systematic Reviews of Interventions [11, 12]. Relevant studies were identified by an online electronic search on July 25, 2017 using PubMed, EMBASE, Web of Science and the Cochrane library. The search strategies were developed by two authors (AWP and NCG) in consultation with a librarian using medical subject heading (MeSH) and text words related to vulvar cancer, local recurrence and prognostic factors, see Supplementary 1.

To ensure completeness of included references, we scanned the reference lists of the included studies or relevant reviews identified through the search. See Figure 1 for the PRISMA flow diagram.

Eligibility criteria and data extraction

Eligible study designs for inclusion were; randomized controlled trials, controlled clinical trials, case-control studies, cross-sectional studies and cohort studies. The eligibility criteria were: studies evaluating (1) the association between clinicopathologic and cell biologic variables and local recurrence of vulvar squamous cell carcinoma (2) including patients above the age of 18 years and (3) written in English. Two review authors (AWP and NCG) independently screened the titles and abstracts retrieved by the search. Two review authors (AWP and NCG) independently decided whether these articles met our inclusion criteria. Data were extracted independently in duplicate by two review authors (AWP and NCG) from eligible studies using a data extraction form designed and pilot tested. Any disagreements were resolved with a third review author (MHO or JAH).

Assessment of quality of included studies

The Newcastle-Ottawa quality assessment Scale for cohort studies was used to assess the quality of each included study independently by two review authors. This is a validated tool in assessing the quality of non-randomized studies [13]. This scale measures aspects of methodology in cohort studies and consists of eight questions concerning three areas (1) the selection of the study groups (four stars), (2) the comparability of cohorts (two stars), and (3) the outcome of study and adequacy of follow-up (three stars). Each study can be rewarded with a maximum of nine stars.

Dealing with missing data

When there were missing data, we contacted the original authors of the studies to obtain the relevant missing data.

Data analyses

Local recurrence was defined as any newly diagnosed invasive squamous cell carcinoma located on the vulva. In patients with simultaneous local and groin and/or distant recurrences, these local recurrences were also included. To estimate the incidence of local recurrences over time, the local recurrence rate for each individual study was displayed in a graph in relation to the length of follow-up. Subanalyses, including for studies counting › 100 patients were performed. To evaluate whether a prognostic factor was associated with an increased incidence of local recurrence, risk differences (RDs) with 95% confidence intervals (95% CIs) were estimated for each individual study and each risk factor.

Due to the differences in study design (e.g. study population, treatment regimens (including adjuvant (radio)therapy), FIGO stage) of the included studies, homogeneity could not be assumed and therefore no meta-analyses were performed. All authors of studies that did not report sufficient data to calculate RDs were contacted to obtain the relevant missing data to calculate the RDs, as described above. If these data were not provided, the hazard ratios (HRs) of the original report were presented separately. Statistical analysis was performed using Review Manager version 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) and IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA).

RESULTS

Study selection

The primary search on July 25, 2017 identified 2259 publications by electronic database searching, while another 17 publications were identified by hand searching. Twenty- three studies met our inclusion criteria; one study was excluded because the authors excluded patients with a positive margin from analysis and did not report sufficient data on other prognostic factors for local recurrence [14]. See Figure 1 for the PRISMA flow diagram.

Characteristics of included studies

Data from 3657 patients from 22 studies were included in this review. See Table 1 ‘Characteristics of included studies’.

Quality assessment

Using the Newcastle-Ottawa score, the included studies scored between three and eight stars with a median of 6 stars (range 3-8). See Table 1 ‘Characteristics of included studies’ for awarded stars for each individual study. Overall, the lowest number of stars was rewarded for comparability, due to a lack of a control group for the studied prognostic factors in the included studies.

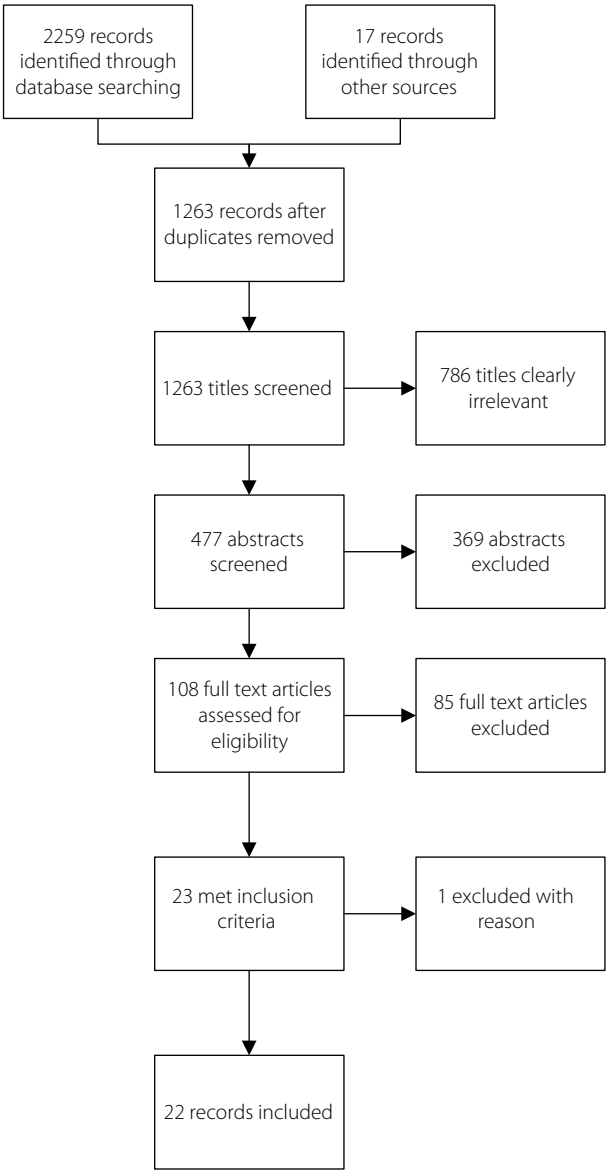

Figure 1 PRISMA flow diagram.

Local recurrence rate

The local recurrence rate by the median follow-up time is displayed for all studies that included > 100 patients, see Figure 2. The estimated median local recurrence rate per year was 4%. The longer the duration of the follow-up, the more patients developed a local recurrence of vulvar carcinoma. See Supplementary 2 for the local recurrence rates by the duration of follow-up of all included studies.

Prognostic factors for local recurrence

Age

Age ≥75 years as a possible prognostic factor was reported by three studies [15-17]. Two studies reported that increasing age was related with an increased risk for local recurrence (age ≥75 as compared to age <75: HR 1.93 (95% CI 0.92-4.07) [16] and a 2% increase in the incidence of local recurrence for each additional year of age [15]. Another study reported that increasing age was related with a decreased risk for local recurrence (age ≥ 75 as compared to age <75; HR 0.35 (95% CI 0.17-0.73) [17].

Pathologic tumor free margin status

Eleven studies reported local recurrences in relation to pathologic tumor free margin distance [8, 9, 17-25], see Figure 3A. All studies except for one used a cut-off value of 8 mm. Six studies reported a decreased risk for local recurrence in patients with a pathologic tumor free margin ≥8mm versus patients with a margin <8mm [9, 17, 20-23]. Five studies showed no difference in the frequency of local recurrences between the two margin groups [8, 18, 19, 24, 25]. Of these five studies, only two reported whether the analyzed margin distance was after re-excision or not .

Four studies reported HRs for local recurrence by margin distance, but none of them showed a relation between margin and frequency of local recurrence [15, 17, 20, 24].

Type of surgery

Eight studies reported no effect when comparing radical vulvectomy versus wide local excision as a possible prognostic factor [9, 17, 19, 20, 26-29]. See Figure 4A.

Vulvar lichen sclerosus

The association of lichen sclerosus and local recurrence was analyzed in five studies [16, 17, 19, 20, 22]. Two studies reported more local recurrences in patients with lichen sclerosus, while no difference in number of patients with local recurrence was reported in four studies [16, 19, 20, 22], see Figure 4B. It was often not reported by the studies how the presence of lichen sclerosus was objected (retracted from patient files or by reviewing pathology slides). One study with reviewed pathology slides reported significantly more local recurrences in patients with lichen sclerosus [17].

Table 1 Characteristics of included studies.

Study	Design, inclusion period	No. of patients	FIGO stage (year)	Duration follow-up (months)		NOS quality score overall
				Mean (SD)	Median (range)	
Arvas 2005[26]	Retrospective multicenter 1989-2002	92	I-IV (1995)	33.48 (24.73)		★★★☆☆☆☆☆
Baiocchi 2015[19]	Retrospective unicenter 1980-2013	205	NR		78 (1-318)	★★★★★★★☆☆
Bogani 2014[20]	Retrospective unicenter 1990-2013	101	I-IV (2009)	37.6 (22.1)		★★★★★☆☆☆☆
Chan 2007[21]	Retrospective multicenter 1984-2002	90	I-IV (NR)		57.5 (2-185)	★★★★★☆☆☆☆
De Hullu 2002[9]	Retrospective unicenter 1982-1997	253	TNM classification; tumors confined to the vulva		110 (3-220)	★★★★★★★☆☆
Fonseca 2000[22]	Retrospective unicenter 1987-1997	42	IB-IVA (1995)	36.9	(2-112)	★★★★★★☆☆☆
Gadducci 2012[30]	Retrospective multicenter 1995-2010	87	I-IV (1988)		61.4 (survivors)	★★★☆☆☆☆☆☆
Heaps 1990[23]	Retrospective multicenter 1957-1985	135	I-IV (1988)	NR		★★★★★☆☆☆☆
Hoffmann 1992[27]	Retrospective unclear 1980-1990	90	I-III (NR)	48	(12-108)	★★★★★☆☆☆☆
Leonard 2016[32]	Retrospective Unicenter 2001-2008	201	NR	>60		★★★★★☆☆☆☆
Lingard 1992[31]	Retrospective unicenter 1980-1990	90 (77 surgically treated)	I-III (1969)	23.2	(1-90)	★★★★★★☆☆☆
Maggino 2000[28]	Prospective multicenter 1980-1994	502	I-IV (NR)	NR		★★★★☆☆☆☆☆
Nooij 2016[24]	Retrospective unicenter 2000-2012	148	I-IV (2009)		42 (0-174)	★★★★★★★☆☆

Podratz 1982[29]	Retrospective unicenter 1955-1975	224	I-IV (1971)		(36-276)	★★★☆☆☆☆☆
Qvick 2017[33]	Retrospective multicenter 1983-2008	123	I-IV (1994)	NR		★★★★★☆☆☆
Sznurkowski 2010[17]	Retrospective unicenter 1998-2001	59	I-IV (1996)		48 (4-200)	★★★★★★★★☆
Tantipalakorn 2009[18]	Retrospective unicenter 1987-2005	121	I, II (1994)		84	★★★★★★★☆☆
Te Grootenhuis 2016[6]	Prospective multicenter 2000-2006	377	I-II (1994)		105 (0-179)	★★★★★★★☆☆
Woelber 2011[8]	Retrospective unicenter 1998-2008	102	I-IV (NR)		31 (1-105)	★★★★★★★☆☆
Woelber 2016[25]	Retrospective multicenter 1998-2008	289	IB – IV (1994)		35.1 (0.2-141.2)	★★★★★★☆☆☆
Woolderink 2006[16]	Retrospective multicenter 1985-1999	125	I-IV (1996)		42 (1-184)	★★★★★★★★☆
Yap 2016[15]	Retrospective unicenter 2000-2008	201	I-IV (1998)		80.4	★★★★★★☆☆☆

Footnote: FIGO: The International Federation of Gynecology and Obstetrics NOS: Newcastle-Ottawa score

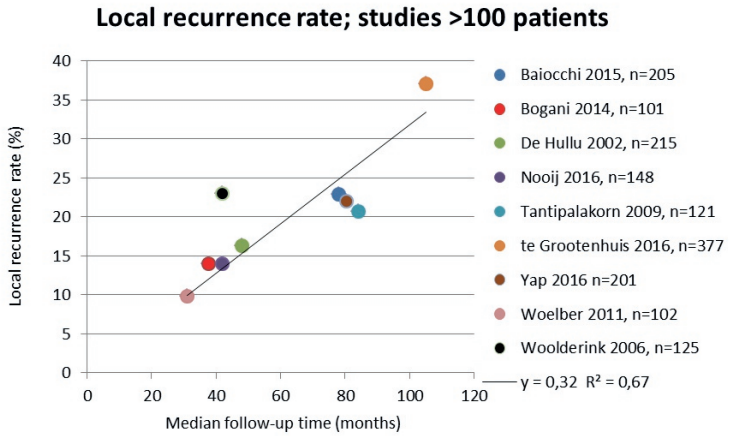

Figure 2 Local recurrence rate by duration of follow-up time

Footnote: displayed for studies including >100 patients. Woelber *et al.* 2016[25] was not included in this analysis because this study only included lymph node negative patients. Maggino *et al.* 2000[28] did not report a (median) follow-up time and could therefore not be included in this analysis.

Regarding studies reporting HRs; in one study the risk for local recurrence in patients was increased in patients with vulvar lichen sclerosus (univariate HR 3.39 (95% CI 1.80-6.38))[15], while three more studies reported no effect [16, 17, 20].

Groin lymph node metastases

Nine studies reported on the prognostic relevance of the presence of groin lymph node metastases (including a positive SLN) and/or number of groin lymph node metastases at primary treatment on local recurrence [6, 15-17, 19, 20, 22, 24, 30]. One study reported more local recurrences in patients with groin lymph node metastasis [22], six studies showed no effect of the presence of groin lymph node metastasis [6, 16, 17, 19, 20, 30], see Figure 3B.

Univariate analysis was performed in seven studies; three studies reported a significant effect whereas Nooij *et al.* and Te Grootenhuis *et al.* found more local recurrences in patients with positive lymph nodes (HR 2.73 (95% CI 1.15-6.51)) respectively (local recurrence rate SLN-negative patients 36.4% versus 46.4% for SLN-positive at 10-years (p = 0.03)) [6, 24]. Contradictory results were found by Sznurkowski *et al.*, who observed more local recurrences in patients with negative lymph nodes (HR 0.23 (95% CI 0.11-0.52)) [17].

The prognostic relevance of the number of lymph node metastases for local recurrence was reported in two studies [22, 30]. Fonseca *et al.* showed a significant decreased risk

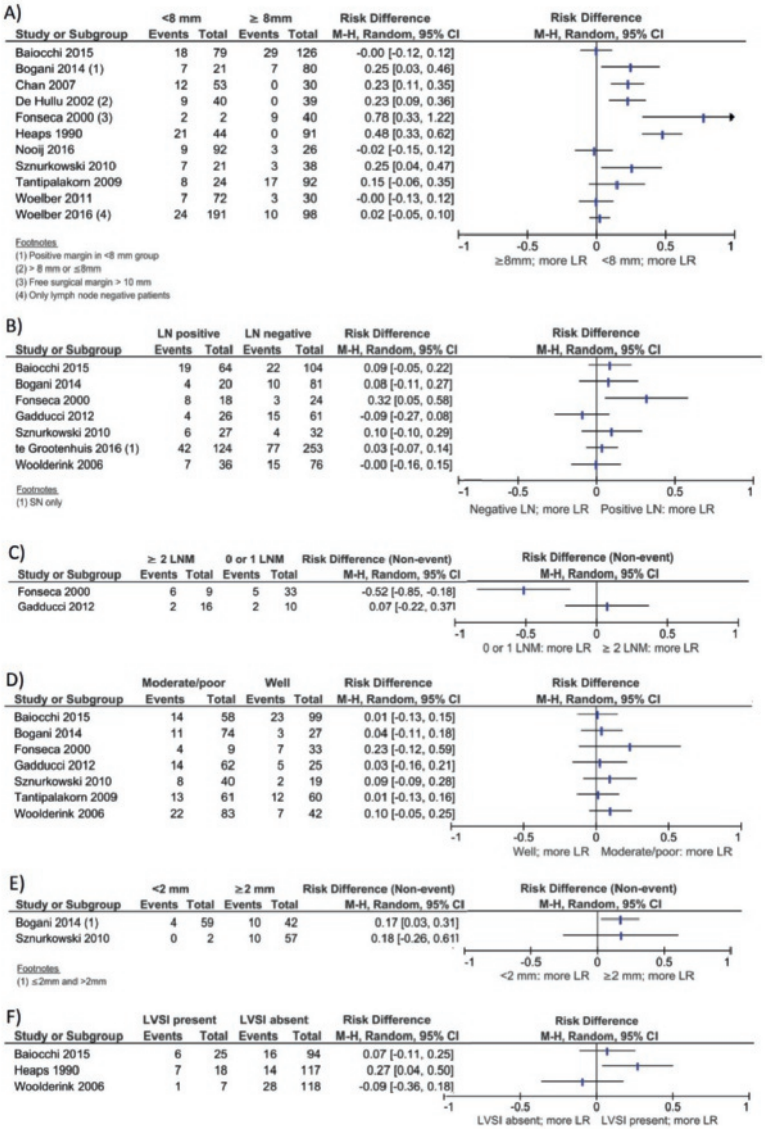

Figure 3 Risk difference for local recurrence by different pathological factors

A) Risk difference for local recurrence by margin distance, B) Risk difference for local recurrence by presence of lymph node metastases, C) Risk difference for local recurrence by the number of lymph node metastases, D) Risk difference for local recurrence by grade of differentiation, E) Risk difference for local recurrence by depth of invasion, F) Risk difference for local recurrence by presence of lymphovascular space involement

Footnote: mm: millimeters, LR: local recurrence, LN: lymph nodes, SLN: sentinel lymph node, LNM: lymph node metastases, LVSI: lymphovascular space involvement

for local recurrence in patients with none or one lymph node metastasis versus patients with two or more lymph node metastases (RD -0.52 (95% CI -0.85, -0.18)), see figure 3C.

Grade of differentiation

The prognostic value of tumor grade for local recurrence is displayed in Figure 3D. None of the studies showed evidence for a relation between grade and number of local recurrences.

Tumor size

Four studies reported the risk for local recurrence by tumor size and divided tumor size into two groups; tumor measuring ≤4 cm or › 4 cm (not specified whether the clinical or pathological tumor size was used). None of these four studies reported an effect of tumor size on the risk for a local recurrence, as shown in Figure 4C [17, 19, 20, 30].
Two studies reported that increasing tumor size (as a continuous variable) did not increase the risk for local recurrence (HR 1.08 95% CI 0.89, 1.30 [19], HR 0.91 (95% CI 0.65, 1.28) [17] .

Tumor focality

The risk for local recurrence as reflected by the RD showed no evidence of effect, see Figure 4D. One study showed a higher risk for local recurrence in patients with multifocal disease in the multivariate analysis using patients with unifocal disease as a reference (HR 2.98 (95% CI 1.08, 7.26) [17].

Depth of invasion

Two studies reported the risk for local recurrence by the depth of invasion of the tumor [17, 20]. One study showed more local recurrences in patients with a tumor with a depth of invasion ›2 mm, see Figure 3E [20]. Another study reported the prognostic influence of depth of invasion on local recurrence as a continuous variable, but did not report a relation with the risk for local recurrence [16].

Lymphovascular space involvement

The prognostic value of the presence of lymphovascular space involvement (LVSI) in the primary tumor for local recurrence was reported in four studies. One study reported an increased risk for local recurrence if LVSI was present [23]. The other two studies reported no evidence of RD, see Figure 3F [16, 19]. Yap *et al.* reported less local recurrences when LVSI was present, reducing the incidence of local recurrence by 74% (univariate HR 0.28 (95% CI 0.11-0.74), using the absence of LVSI as a reference (HR1.0) [15].

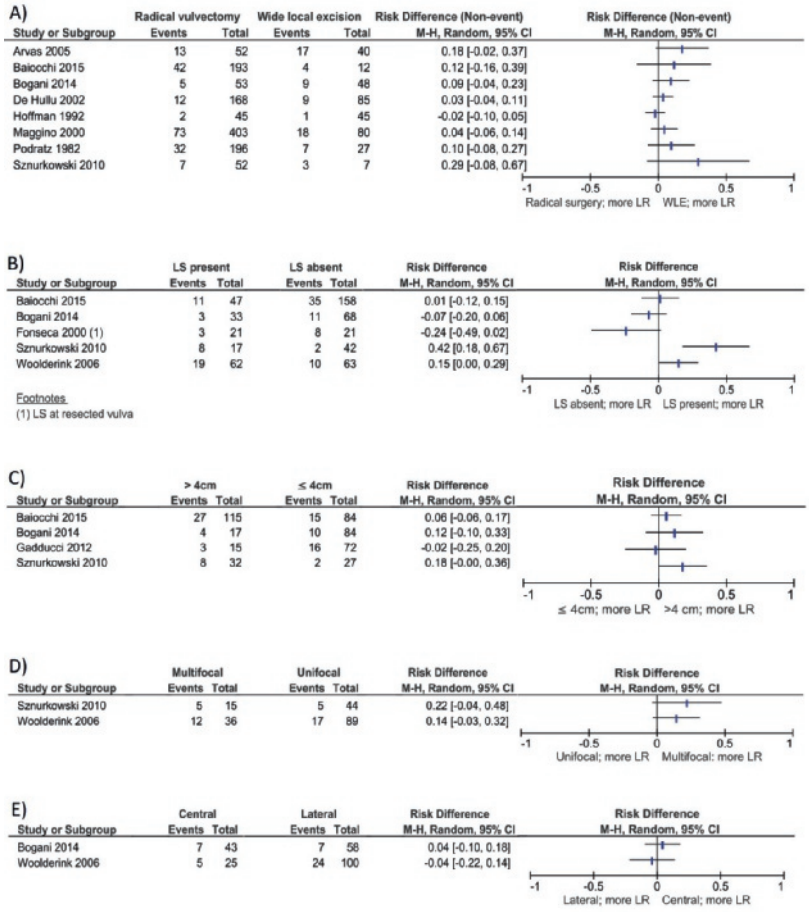

Figure 4 Risk difference of local recurrence by different clinical factors

A) Risk difference for local recurrence by type of surgery, B) Risk difference for local recurrence by presence of lichen sclerosus, C) Risk difference for local recurrence by tumor size, D) Risk difference for local recurrence by tumor focality, E) Risk difference for local recurrence by tumor localization

Footnote: LR: local recurrence, WLE: wide local excision, LS: lichen sclerosus, cm: centimetres

Tumor localization

Bogani *et al.* and Woolderink *et al.* compared the risk for local recurrence in patients with a lateralized tumor versus a centralized tumor; both studies did not find any effect, see Figure 4E [16, 20].

HPV status

Two studies described univariate HRs for local recurrences in relation to the presence of HPV. Yap *et* al. reported more local recurrences in HPV negative patients (HR 2.38 (95% CI 1.15-4.93)) [15], while Nooij *et al.* reported less local recurrences in HPV positive patients (HR 0.24 (95% CI 0.03-1.80)) [24]. In conclusion, both studies reported less local recurrences in HPV positive patients.

Remaining factors

The following prognostic factors were reported by only one study and no evidence of effect was reported; comorbidity, perineural invasion, mucosal involvement, koilocytosis, laterality of lymph nodes, SLN biopsy performed, number of lymph nodes yielded, inguinofemoral lymphadenectomy performed, VIN or VIN3 in margin or around tumor, adjuvant radiotherapy given [16, 18-20, 22, 28, 30, 31].

Leonard *et al.* investigated if DNA methyltransferases (DNMT) expression in the invasive component of the tumor was related to the risk of local recurrence of the vulvar carcinoma. Over-expression of both DNMT3A and DNMT3B was associated with an increased risk of vulvar local recurrence (univariate HR 4.51 (95% CI 1.40, 14.49), HR 5.69 (95% CI 1.17, 27.57) respectively) [32]. Furthermore, Qvick *et al.* reported on the prognostic value of p53 codon 72 polymorphism [33]. A similar local recurrence rate in tumors with arg/arg genotype and in tumors with arg/pro or pro/pro genotype was observed.

DISCUSSION

Summary of main results

Our review shows that for all variables analyzed the prognostic relevance for local recurrence of vulvar carcinoma remains questionable, including pathologic tumor free margin distance, presence of vulvar lichen sclerosus, groin lymph node metastases and a variety of primary tumor characteristics.

Despite the fact that the majority of included studies showed a higher risk for local recurrence in patients with a pathologic tumor free margin distance ‹ 8 mm, it remains unclear if the risk for a local recurrence is also higher when a lower cut-off value is applied. Only two studies investigated the risk for local recurrence for different cut-off values and these studies did not show a difference in risk for local recurrence [24, 25]. Currently, different guidelines on vulvar carcinoma are available, all containing largely consensus based recommendations [10, 34, 35]. In these guidelines, the recommended minimal pathologic tumor free margin in relation to whether further treatment is needed differs between zero mm and ‹ 10 mm, see Table S3. In addition, also in the treatment of cutaneous and head and neck squamous cell carcinoma, different tumor

free margins are recommended compared to vulvar carcinoma. In head and neck squamous cell carcinoma patients, the pathologic free tumor margins have also been at debate for decades. As in vulvar carcinoma, excision is often difficult and limited because of the anatomic site of the tumor. The National Comprehensive Cancer Network (NCCN) guidelines for head and neck carcinoma defines a clear margin as ≥5 mm [36]. In the treatment of cutaneous squamous cell carcinoma, different surgical margins are recommended for low-risk and high-risk lesions. For low-risk lesions, the recommended surgical margin varies between 4-6 mm and for high-risk lesions from 6 to 10 mm [37]. Even though certain risk factors for local recurrence are described in literature, consensus on the specific tumor characteristics defining these low and high-risk lesion has not yet been made, resulting in different definitions in different guidelines [37]. The NCCN guideline defines high-risk lesions as those >10mm occurring in high-risk areas (genitalia, mucosal surfaces, face, and/or neck) or those >20mm in other areas and in case of poorly-defined borders, perineural or vascular invasion, depth of invasion ≥2mm, rapidly growing, moderate or poorly differentiated, site of prior radiotherapy, neurologic symptoms, recurrent lesions or in immunosuppressed patients [38].

From our review it can be concluded that currently available data does not allow real evidence-based medicine. Based on our current review there seems to be no lower limit (apart from involved margins) below which further treatment (either re-excision or adjuvant radiotherapy) to the vulva should be recommended. Considering the impressive morbidity associated with adjuvant radiotherapy to the vulva, the equivocal prognostic impact of margins on local recurrence rate and in general the principle 'primum non nocere' doctors should be reluctant before deciding to expose their patients to these potentially harmful adjuvant therapies.

Adjuvant therapy, including re-excision and/or radiotherapy for a close or positive margin, is a major confounder when it comes to evaluating margin status and local recurrence risk. Unfortunately, only two included studies reported whether the margin distances after the primary excision or after re-excision were used for the analysis [24, 25]. Inevitably this may have introduced bias in most of the original studies, as adjuvant therapy (i.e. re-excision or radiotherapy) most probably will influence the risk for local recurrence.

Regarding adjuvant radiotherapy; most included studies reported the indications and/or number of patients with adjuvant radiotherapy. However, none of them analyzed the subgroup of patients with a close or positive margin with and without adjuvant therapy separately. Adjuvant radiotherapy may result in a lower recurrence risk in the group of patients with a margin <8 mm. In conclusion, not reporting outcome for separate subgroups of patients with or without adjuvant therapy for a close or positive margin, introduced a significant bias and illustrates the lack of uniform reporting on tumor free margin and/or adjuvant therapy as prognostic factors for local recurrence.

Further research is needed in vulvar carcinoma patients to determine the optimal cut-off value for the tumor free margin status and risk for local recurrences. A suitable study design may be a prospective cohort study with sufficient long follow-up, in which pathologic margin distance is measured in a standardized way, adjuvant treatment is given according to protocol and finally in which the margin distance as a prognostic factor is reported in HR or a Kaplan-Meier. Following the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) most certainly will help to improve the quality of future studies [39].

Our review indicates an estimated local recurrence rate per year of 4% with no plateauing and therefore a higher local recurrence rate with longer follow-up, see Figure 2. Most included studies reported crude local recurrence rates. By using this method, the local recurrence rate is prone to bias by the fact that at time of diagnosis half of the patients is aged over 60 years and therefore more likely to die during follow-up. Reporting crude recurrence rates will lead to underestimation of the real local recurrence rate. As Figure 2 shows, the longer the follow-up, the higher the incidence rate for local recurrences. Thus, studies with a short follow-up time are more likely to underreport the local recurrence rate. Our review shows that even many years after primary treatment, recurrences still occur, which is important to realize in light of follow-up schedules and patient counseling.

Current literature on vulvar cancer artificially discriminates between two kinds of local recurrences; a so-called 'true' local recurrence arising from residual cancer cells after an irradically treated primary tumor and a 'de novo' recurrent tumor arising from a premalignant lesion.

First, based on the localization of the recurrence; a 'de novo' tumor has been defined as >2 cm away versus a true local recurrence as ≤2 cm from the primary tumor [15, 18]. Second, based on the time to recurrence; a 'true' local recurrence has been defined as ≤2 years and a 'de novo' tumor >2 years after primary treatment [6, 24]. As shown previously, local recurrences occurring >2 years after primary treatment tend to have a better disease-specific survival (p=0.05) [6]. To really distinguish a 'true' local recurrence from a 'de novo' recurrence genetic relationship and molecular profiling analysis should be performed. The current division based on e.g. distance of recurrence to primary tumor site or time to recurrence is too arbitrary, not reproducible and should be abandoned.

In light of the possible prognostic impact of a premalignant lesion adjacent to the malignant lesion, it also seems unjustified to divide 'de novo' and true local recurrences based on their location.

Unfortunately, only little research is performed on this subject. Adjacent Vulvar Intra-epithelial Neoplasia (VIN) has been investigated in few studies, different classification systems for VIN (dVIN, VIN I-III) have been used and therefore results cannot be compared. Six studies investigated local recurrence rates for patients with and without

vulvar lichen sclerosus and found conflicting results [15-17, 19, 20, 22]. Only two studies, observed more local recurrences in patients when lichen sclerosus was present [15, 17]. More extensive removal of (adjacent) premalignant lesions may also explain a lower risk for local recurrence in patients treated by radical vulvectomy, see Figure 4A.
Six out of seven studies reported groin lymph node metastasis as a prognostic factor for local recurrent disease (Figure 3B). In contrast to these studies; Gadduci *et al.* were the only to find more local recurrences in patients with negative lymph nodes. However, they reported a very low two year overall survival rate (38%) for the patients with positive lymph nodes, which may have caused the lower local recurrence rate in these patients, as their follow-up time was shorter [30].

Strengths

Our study is the first systematic review on prognostic factors for local recurrences in vulvar squamous cell carcinoma, summarizing all currently available evidence on prognostic factors for local recurrence of vulvar carcinoma and applying the PRISMA guidelines. Furthermore, quality scores (Newcastle-Ottawa) were calculated for each included study, thereby minimizing the risk of bias. By calculating a RD and displaying these data in forest plots, the outcomes of this review are clarified and more easy to interpret.

Limitations

Because of the low incidence of vulvar carcinoma the included studies were very heterogeneous and therefore meta-analysis could not be performed.

Conclusions

Current evidence on prognostic factors for local recurrence of vulvar cancer is not sufficiently robust to allow evidence-based medicine. More research of higher quality is needed to identify clinicopathologic and cell biologic factors prognostic for local recurrence of vulvar carcinoma. More accurate identification of patients at high risk for local recurrence should lead to more individualized treatment and/or follow-up protocols in these patients.

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SUPPLEMENTARY DATA

Supplementary 1 Search strategies.

1a. Pubmed search

(Vulvar neoplasms[mesh] OR (Vulva*[tiab] AND (neoplasm*[tiab] OR cancer*[tiab] OR tumor*[tiab] OR tumour*[tiab] OR carcinoma*[tiab])))
AND
((“Neoplasm Recurrence, Local”[Mesh] OR Recurr*[tiab] OR relaps*[tiab] OR reoccur*[tiab]))
AND
((“Prognosis”[Mesh:noexp] OR “Disease-Free Survival”[Mesh] OR “Risk Factors”[Mesh] OR Time Factors[Mesh] OR Epidemiology[subheading] OR Survival Rate[MeSH] OR Prognos*[tiab] OR predict*[tiab] OR risk factor*[tiab]))

1b. EMBASE search

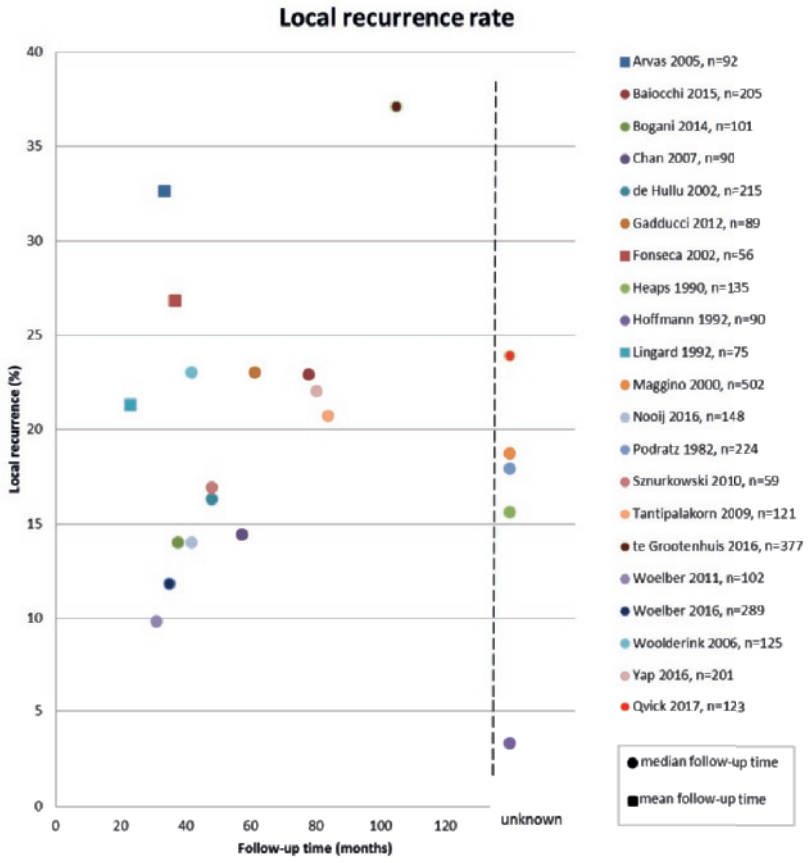
(Exp vulva cancer/ OR exp vulva carcinoma/ OR exp vulva tumor/ OR ((Vulva*.ti,ab,kw.) AND (neoplasm*.ti,ab,kw. OR cancer*.ti,ab,kw. OR tumor*.ti,ab,kw. OR tumour*.ti,ab,kw. OR carcinoma*.ti,ab,kw.)))
AND
Exp tumor recurrence/ OR exp cancer recurrence/ OR Recurr*.ti,ab,kw. OR relaps*.ti,ab,kw. OR reoccur*.ti,ab,kw. OR recidiv*.ti,ab,kw.
AND
Exp prognosis/ OR exp cancer prognosis/ OR exp local recurrence free survival/ OR exp disease-Free Survival/ OR exp risk factor/ OR Prognos*.ti,ab,kw. OR predict*.ti,ab,kw. OR risk factor*.ti,ab,kw.

1c. Web of Science

“Vulva* neoplasm*” OR “Vulva* cancer*” OR “Vulva* tumor*” OR “Vulva* tumour*” OR “Vulva* carcinoma*” OR “squamous cell carcinoma of the vulva*” OR “vulva* squamous cell carcinoma*”
AND
Recurr* OR relaps* OR reoccur* OR recidiv*
AND
“Disease* Free Survival” OR Prognos* OR predict* OR “risk factor*“

1d. Cochrane search

Vulva*:ti,ab,kw AND (neoplasm*:ti,ab,kw OR cancer*:ti,ab,kw OR tumor*:ti,ab,kw OR tumour*:ti,ab,kw OR carcinoma*:ti,ab,kw)
AND
Recurr*:ti,ab,kw OR relaps*:ti,ab,kw OR reoccur*:ti,ab,kw
AND
Prognos*:ti,ab,kw OR predict*:ti,ab,kw OR (risk factor*):ti,ab,kw



Supplementary 2 Overall local recurrence rate by duration of follow-up

Footnote: no data for Leonard *et al.* 2016[32] available, Woelber *et al.* 2016[25]; only patients with negative groin lymph nodes.

Supplementary 3 Recommendations in different guidelines concerning tumor free margin distance in the treatment of vulvar carcinoma.

Guideline	Recommended surgical tumor free margin (mm)	Recommended pathologic tumor free margin(mm)	Adjuvant treatment indicated if pathologic tumor free margin (mm);	Adjuvant treatment consist of;
The Royal College of Obstetricians & Gynaecologists[34]	≥ 15	‹ 10	‹ 10 and/or should be considered if the remainder of the vulva is affected by atypical skin (lichen sclerosus or VIN)	'appropriate to perform a further local resection'
National Comprehensive Cancer Network[10]	10-20	‹ 8	‹ 8	're-resection and/or adjuvant therapy, must be considered and individualized in each patient'
European Society of Gynaecological Oncology[35]	≥ 10	-	0 and/or consider in case of VIN	' re-excision is treatment of choice'

Footnote: VIN: Vulvar intraepithelial neoplasia


5

Margin status revisited in vulvar squamous cell carcinoma

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ABSTRACT

Objective To determine the incidence of local recurrence of vulvar squamous cell carcinoma in relation to tumor- and/or precursor lesion free pathologic margins.

Methods Consecutive patients with primary vulvar squamous cell carcinoma surgically treated in two Dutch expert centers between 2000 and 2010 were included. All pathology slides were independently reviewed by two expert gynecopathologists, and local recurrence was defined as any recurrent disease located on the vulva. Time to first local recurrence was compared for different subgroups using univariable and multivariable Cox-regression analyses.

Results In total 287 patients with a median follow-up of 80 months (range 0-204) were analyzed. The actuarial local recurrence rate ten years after treatment was 42.5%. Pathologic tumor free margin distance did not influence the risk on recurrence (HR 1.03 (95% CI 0.99-1.06)), neither using a cutoff of eight, five, or three millimeters. Multivariable analyses showed a higher local recurrence rate in patients with dVIN and LS in the margin (HR 2.76 (95% CI 1.62-4.71)), in patients with dVIN in the margin (HR 2.14 (95% CI 1.11-4.12)), and a FIGO stage II or higher (HR 1.62 (95% CI 1.05-2.48)).

Conclusions Local recurrences frequently occur in patients with primary vulvar carcinoma and are associated with dVIN (with or without LS) in the pathologic margin rather than any tumor free margin distance. Our results should lead to increased awareness among physicians of an ongoing risk for local recurrence and need for life-long follow-up. Intensified follow-up and treatment protocols for patients with dVIN in the margin should be evaluated in future research.

BACKGROUND

In patients treated for early-stage vulvar squamous cell carcinoma, local recurrences are reported in up to 40% in the first 10 years after primary diagnosis [1]. A recent systematic review from our group estimated an annual local recurrence rate of 4% without plateauing despite adequate treatment [2]. In literature, data on prognostic factors related to local recurrences are mostly limited to classical clinico-pathologic factors. These data are heterogeneous and not sufficiently robust to propose individualized treatment and follow-up guidelines related to the risk on local recurrences [2].

Even though the current surgical approach has less morbidity than before, radical surgery of the vulva is still mutilating. Currently, the recommended surgical tumor-free margin distance varies between different guidelines, ranging between one to two centimeters [3-5]. The pathologic tumor margin distance with cut-off value of eight millimeters has frequently been challenged as a prognostic factor. However, studies investigating a lower cut-off value are scarce, retrospective and without proper central pathologic review. Therefore data available so far are insufficient to draw conclusions on which pathologic tumor free margin distance is safe without increasing the local recurrence rate [6, 7].

Besides the tumor-free margin distance, vulvar precursor lesions in the skin adjacent to the tumor and/or in the margin could be of prognostic significance. Two different pathways with their own precursor lesions have been identified so far in the development of vulvar squamous cell carcinoma; the first and most common pathway is associated with lichen sclerosus (LS) and differentiated vulvar intraepithelial neoplasia (dVIN). The second pathway is caused by a persistent human papillomavirus (HPV) infection with high-grade squamous intraepithelial lesions (HSIL) as associated precursor [8]. Data from recently published studies indicate that the presence of LS in the resection specimen of vulvar carcinoma may strongly increase the risk of local recurrences [9, 10].

Currently no stratification of vulvar carcinoma patients with respect to their risk for local recurrence is possible. However, identification of patients at such low risk that they can be discarded from follow-up, for example after two or five years, would have significant clinical benefit. Simultaneously for high-risk patients new strategies might be developed and evaluated to prevent local recurrences.

The main aim of this study was to determine the incidence of local recurrence of vulvar squamous cell carcinoma, in a clinically well-defined consecutive patient series from two expert centers. The secondary aims were to assess the relation of local recurrence to tumor- and/or precursor lesion free pathologic margins determined by extensive pathology review, and based on these results to identify different risk groups, allowing future individualized treatment and follow-up strategies.

METHODS

This study is reported in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) [11].

Patients

Consecutive patients with vulvar squamous cell carcinoma treated at the Radboud university medical center and University Medical Center Groningen from January 2000 – December 2010 were eligible for analysis. Both centers are expert centers for the treatment of vulvar carcinoma. Eligibility criteria for this study were: primary diagnosis of vulvar squamous cell carcinoma and primarily surgically treated at one of the two participating centers. Patients who suffered from multifocal disease, or patients who received neo-adjuvant chemotherapy and/or radiotherapy, definitive (chemo) radiation or palliative treatment were not included.

Clinical data of all patients treated for vulvar carcinoma in both centers were prospectively stored in a database and completed by retrospective review of the patient charts. We identified eligible patients for this study from this database. To ensure completeness, we searched the Dutch nationwide registry of histopathology and cytology (PALGA), which resulted in nine additional patients in the two centers.

Treatment and follow-up

The surgical treatment of the vulva consisted of either a radical vulvectomy or wide local excision of the tumor. For a wide local excision, the intention was to obtain surgical tumor-free margins of at least 10 mm. In patients with a macro-invasive tumor (depth of invasion > 1 mm), a sentinel node procedure and/or an inguinofemoral lymphadenectomy was performed. Adjuvant therapy with re-excision was recommended in patients with a tumor-positive margin. When re-excision was not possible, adjuvant radiotherapy to the vulva was recommended. In patients with a pathologic tumor free margin of < 8 mm close follow-up was performed. When patients had an indication for radiotherapy to the groins, adjuvant radiotherapy on the vulva was considered in patients with pathologic tumor free margin distance of < 8mm. Adjuvant radiotherapy to the groin was indicated for patients with ≥ 2 metastatic lymph nodes, or in case of extra nodal growth. From 2000 until 2006, patients from both our centers participated in the GROningen INternational Study on Sentinel Nodes in Vulvar cancer (GROINSS-V) I study, while from 2006 onwards patients participated in the GROINSS-V II study. Long-term follow-up data of patients with early-stage disease who were included in the GROINSS-V I study have been published previously [1]. After treatment, patients were examined routinely every two to three months during the first two years after completion of primary treatment, every six months during the third and fourth year and yearly thereafter.

Histopathologic review

For this study, all formalin-fixed and paraffin-embedded and hematoxylin and eosin stained slides, were reviewed in a standardized way by two independent expert gynecopathologists (JB and HH), blinded for the results of treatment and follow-up data. Eight test cases were analyzed by both gynecopathologists independently,after which agreement scores for these eight cases were calculated and a consensus meeting was organized to reduce the interobserver variability. Before the consensus meeting percentage of agreement was 62-66% for the assessment of the presence of LVSI, differentiation grade and the presence of LS in the pathologic margin, 100% for the assessment of LS, HSIL and/or dVIN adjacent to the tumor and 100% for the assessment of HSIL and dVIN in the pathologic margin. The intraclass correlation coefficient for the smallest pathologic margin was 0.98 and for the depth of invasion 0.94. After the consensus meeting, interobserver variety was minimal for all variables analyzed. Histopathological review of the included patients was performed by one of the two expert gynecopathologists and included the following variables: tumor type, pathologic tumor free margin distance for the basal and lateral margin separately, presence of low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), lichen sclerosus (LS) or differentiated vulvar intraepithelial neoplasia (dVIN) adjacent to the tumor and/or in the pathologic margin (according the ISSVD classification [8]), depth of invasion, tumor thickness, tumor diameter, presence of lymph-angio-invasion, growth pattern and grade of differentiation. If re-excision was performed, both the slides of the primary excision and re-excision were reviewed.

Data handling

All data were entered in an anonymous database using Castor EDC in which patient identity was protected by study-specific unique patient numbers [12]. The codes of these specific numbers were only known to two dedicated data managers for each center separately. The use of these procedures combined with the fact that patients did not object against use of their clinical data or tumor material, meant that, according to Dutch law, no further patient or IRB approval was needed.

Endpoints

The primary endpoint was time to local recurrence of vulvar carcinoma. Local recurrence was defined as any newly diagnosed invasive squamous cell carcinoma located on the vulva, and time to local recurrence was defined as the period of time in months from the date of primary surgery to the date at which recurrence was identified by histopathology. The end of follow-up was defined as date of last follow-up or date of death. Patients were reported lost to follow-up if no information on the last 24 months was available at time of data collection. Follow-up data were collected until January 1st, 2018. The median follow-up time was 80 months (range 0-204 months).

Definitions

The tumor specimens were formalin-fixed and paraffin-embedded (FFPE) in tissue blocks and all pathologic tumor free margin distances were measured on hematoxylin and eosin (H&E) stained slides from these blocks. Multiple sections at the tumor edges and margin were performed, and all reviewed in order to detect the smallest pathologic margin. The pathologic tumor free margin distance was defined as the distance in millimeters from the tumor edge to the end of the specimen, measured along the epithelium after formalin fixation using a ruler. All lateral tumor free margins were measured. Besides, the basal tumor free margin was measured. We determined the closest pathologic tumor free margin by taking into account both the lateral and basal margins. In case re-excision was performed, the closest margin after re-excision was assessed. The depth of invasion was measured from the epithelial-stromal junction of the most superficial adjacent dermal papillae to the deepest point of invasion as recommended by The International Society of Gynecological Pathologists (ISGYP) and The International Federation of Gynecology and Obstetrics (FIGO) [13]. Regarding the precursor lesions; we noted the presence of these lesions as 1) adjacent to the tumor; but not in the pathologic margin or as 2) precursor lesion in margin; located in the pathologic margin. For the variable presence of precursor lesion in the pathologic margin, the latter had to be present. For the variable presence of precursor lesion in the excised specimen either one of the two previously described variables; a precursor lesion adjacent to the tumor or in the margin or both were present. The following patient, tumor and treatment characteristics were collected: age, FIGO stage (2009), TNM stage, tumor localization, treatment given (primary and adjuvant), histopathologic outcomes and follow-up data.

Statistical analysis methods

Continuous variables were summarized using the median and range, discrete variables were described by frequencies. The local recurrence rate was determined using the Kaplan-Meier method. Censoring was applied to patients alive without local recurrence at last follow-up and patients who died. Time to first local recurrence was calculated from the date of primary surgery and compared for each prognostic factor performing univariate Cox-regression analysis; hazard ratios (HR) with 95% confidence intervals (CI) were presented. A p-value of < 0.05 was considered to be statistically significant. Variables that had a p-value < 0.200 or were considered clinically relevant were incorporated in a multivariable Cox-regression analysis. The Akaike information criterion (AIC) and Bayesian information criterion (BIC) values were analyzed to compare the relative quality of the different Cox-regression models. A lower AIC of BIC indicates a better fit of the model compared to the other models. Together with the significance of the variables, the multivariable model with the best fit was chosen. Data analysis was performed using SPSS software (version 25.0, Armonk 2017) [14] and the statistical software R (version 3.5.0), with the survival package.

RESULTS

During the study period, 435 patients were primarily surgically treated for vulvar carcinoma. For a variety of reasons, 148/435 patients were excluded, see flowchart in Supplementary Figure 1. In total, data from 287 patients were analyzed. Median age was 73 years (range 26-100), and all TNM and FIGO stages were represented, except FIGO stage IVB. Fifty-two patients were lost to follow-up for at least two years at time of data collection; median follow-up of these patients was 64 months (range 0-196). Clinical and histopathologic characteristics of the study population are listed in Table 1, and did not differ between the two centers.

The actuarial local recurrence rates five and ten years after primary treatment were 28.3% and 42.5%, respectively, see Figure 1. This rate did not differ between patients from both treatment centers individually: 5 and 10-year local recurrence rates were 26,0% and 30,5% and 42,6% and 43,5% respectively (p = 0.679). Median time to local recurrence was 32 months (range 0-202 months) and this did not differ significantly per precursor lesion subgroup (p = 0.08).

Pathologic margin in relation to local recurrences

The pathologic tumor free margin distance had no effect on the local recurrence rate (continuous HR 1.03 (95% CI 0.98-1.06)). No differences in local recurrence rate were observed, neither for the cut-off values ≥8 mm versus <8 mm, nor for different cut-off values (3-8 mm) (p = 0.308). Exclusion of patients with adjuvant radiotherapy on the vulva also did not indicate more local recurrences in relation to a smaller tumor-free margin distance. Because of small subgroups, patients were categorized using the cutoff point of eight, five and three millimeters, as shown in Table 2 and Supplementary Table 1.

The local recurrence rate ranged from 28.1% for patients with HSIL, 30.7% for patients with no precursor lesion, 44.2% for patients with LS, 44.8% for patients with dVIN, and 76.4% for patients with both LS and dVIN in the resection margin 10 years after treatment, respectively (See Figure 2). Univariable analyses of all included patients using binary variables showed that dVIN and/or LS in the margin, was associated with more local recurrences compared to no dVIN and/or LS in the margin (dVIN and LS present; HR 2.58 (95% CI 1.55-4.32); dVIN present HR 2.39 (95% CI 1.54-372), LS present HR 1.56 (95% CI 1.02-2.39), see Table 2. There was no difference in local recurrence rate when HSIL was present in the margin compared to no HSIL in the margin.

Figure 2 shows that patients with dVIN in the margin, with or without LS, have significant higher local recurrence rates compared to patients without any precursor lesion in the margin (HR 3.32 (95% CI 1.79-6.16) and HR 2.28 (95% CI 1.10-4.71) respectively). Furthermore, within the subgroup of patients with dVIN in the excised specimen, the local recurrence rate was higher in patients with dVIN located in the margin

Table 1 Clinical and histopathologic characteristics of the study population.

	Median (range)	Total 287 patients N (%)
Clinical characteristics		
Age at primary treatment (years)	73 (26-100)	
FIGO stage 2009/ TNM stage		
- IA / T1aN0M0		9 (3)
- IB / T1bN0M0		124 (43)
- II / T2N0M0		5 (2)
- IIIA / T1,2N1a,bM0		70 (24)
- IIIB / T1,2N2a,bM0		13 (5)
- IIIC / T1,2N2cM0		58 (20)
- IVA / T1,2N3M0, T3NanyM0		5 (2)
- IVB / TanyNanyM1		0(0)
- Missing		3 (1)
Local surgery at primary diagnosis		
- Wide local excision		233 (81)
- Radical vulvectomy		46(16)
- Exenteratio posterior		5 (2)
- Skinning vulvectomy		3[a](1)
Groin treatment at primary diagnosis[b]		
- SN		168
- IFL		115
- Primary radiotherapy		1
- Debulking		13
- No treatment		15[c]
Adjuvant therapy		
- Radiotherapy to the vulva		49 (17)
- Re-excision		17 (6)
- Chemotherapy		2 (1)
- None		219 (76)
Status		
- Alive		152 (53)
- Died of vulvar carcinoma		57 (20)
- Died of intercurrent disease		68 (24)
- Died of unknown cause		10 (3)
Histopathologic characteristics		
Tumor diameter[d]	29.5 mm (1.5-130.0)	
Depth of invasion[e]	5.6 mm (0.5 – 25.0)	
Location		
- Central		211 (74)
- Lateral		72 (25)
- Unknown		4 (1)

Table 1 Continued.

	Median (range)	Total 287 patients N (%)
Histopathologic characteristics		
Grade of differentiation		
- Grade 1		83 (29)
- Grade 2		127 (44)
- Grade 3		74 (26)
- Not assessed		3 (1)
Growth pattern		
- Spray		105 (36)
- Invasive		96 (34)
- Confluent		69 (24)
- Mixed		7 (2)
- Not assessed*		10 (3)
LVSI		
- No		221 (77)
- Yes		62 (22)
- Not assessed*		4 (1)
Margin after (re)excision[f]	9.0 mm (0 – 35.0)	
- tumor positive		14 (5)
- <3 mm		36 (13)
- <5 mm		59 (21)
- <8 mm		130 (46)
- ≥8 mm		155 (54)
Presence of precursor lesion		
- Lichen sclerosus and dVIN		133 (46)
- dVIN		64 (22)
- Lichen sclerosus		34 (12)
- HSIL		30 (11)
- None		26 (9)
Presence of precursor lesion in margin		
- Lichen sclerosus and dVIN		39 (14)
- dVIN		26(9)
- Lichen sclerosus		104 (36)
- HSIL		15 (6)
- None		103 (36)

[a] Three patients underwent a skinning vulvectomy because of vulvar intraepithelial neoplasia, coincidentally these patients also had invasive squamous cell carcinoma, that was excised sufficiently. [b] Patients undergoing two different groin surgeries are counted in both treatment groups. [c] Nine patients did not receive groin treatment because of a microinvasive tumor, 3 because of comorbidity, 1 wish of the patient, 1 patient had advanced metastatic disease and in 1 case the reason was unknown.[d]Not able to assess in 11 cases.[e]Not able to assess in 4 cases. [f]Not able to assess in 2 cases *Not able to assess due to small tumors.

Abbreviations: SN: sentinel node, IFL: inguinofemoral lymphadenectomy LS: lichen sclerosus, LSIL: low-grade squamous intraepithelial lesions, HSIL: high-grade squamous intraepithelial lesions ,dVIN: differentiated vulvar intraepithelial neoplasia, LVSI: lymph-vascular space invasion.

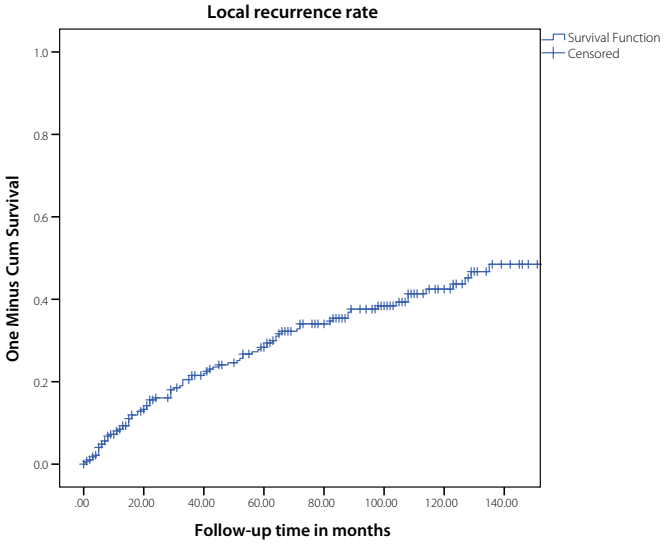


Figure 1 Local recurrence rate

compared to patients without dVIN in the margin but adjacent to the tumor (10- year local recurrence rate 60.5% versus 41.6% respectively, p = 0.002), see Figure 3.

Presence of precursor lesion in the excised specimen in relation to local recurrences

Univariable Cox-regression analyses were performed using a binary variable for the presence of a precursor lesion in the excised specimen. The presence of dVIN, LS or both dVIN and LS in the excised specimen was associated with a higher local recurrence rate (HR 1.80 (95% CI 1.08-2.99) and HR 1.61 (95% CI 1.03-2.52), 1.58 (95% CI 1.04-2.41) respectively) compared to patients without these precursor lesions present. The presence of HSIL was associated with a lower local recurrence rate (HR 0.32 (95% CI 0.14-0.75) compared to patients without HSIL (see Supplementary Table 2).
Univariable Cox-regression analyses dividing patients in five subgroups based on the presence of a precursor lesion in the excised specimen (no precursor lesion, dVIN and LS, dVIN, LS and HSIL) showed no difference in local recurrence rate between the subgroups of precursor lesions and the subgroup without precursor lesions.

Table 2 Clinical and histologic characteristics related to local recurrence (univariable).

	Hazard ratio (95% confidence interval)	
	Whole cohort (n=287)	Vulvar radiotherapy excluded (n=236)
Patient- and treatment characteristics		
Type of local surgery		
- Wide local excision	1.0	1.0
- Skinning vulvectomy	1.90 (0.47-7.77), p=0.369	1.83 (0.45-7.51), p=0.398
- Radical vulvectomy	1.05(0.55-1.97), p=0.887	1.08 (0.52-2.25), p=0.841
- Exenteratio posterior	0.00 (0.00-4.05^{e+204}), p=0.964	0.00 (0.00-4.8^{e+200}), p=0.964
FIGO stage		
- IA, IB, II	1.0	1.0
- IIIA, IIIB, IIIC, IVA	1.45 (0.95-2.21), p= 0.084	1.51 (0.96-2.39), p=0.075
Adjuvant radiotherapy on the vulva		
- No	1.0	-
- Yes	0.84 (0.46-1.54), p=0.569	-
Location		
- Central tumor	1.0	1.0
- Lateral tumor	1.17 (0.73-1.85), p=0.508	1.16 (0.72-1.89), p=0.543
Pathologic margin		
Tumor free margin distance (continuous)	1.03 (0.99-1.06), p=0.153	1.03 (0.98-1.08), p=0.309
Tumor free margin distance		
- ‹8 mm	1.0	1.0
- ≥8 mm	1.25 (0.81-1.93), p=0.308	1.29 (0.79-2.09), p=0.307
Tumor free margin distance		
- ‹5 mm	1.0	1.0
- ≥5 mm	1.13 (0.63-2.05), p=0.678	0.92 (0.44-1.91), p=0.814
Tumor free margin distance		
- ‹3 mm	1.0	1.0
- ≥3 mm	0.93 (0.47-1.85), p=0.831	0.62 (0.25-1.53), p=0.298
Dvin and lichen sclerosus in margin		
- No	1.0	1.0
- Yes	2.58 (1.55-4.32), p‹0.001	2.57 (1.47-4.50), p=0.001
dVIN in margin		
- No	1.0	1.0
- Yes	2.39(1.54-3.72), p‹0.001	2.55(1.58-4.11), p‹0.001
Lichen sclerosus in margin		
- No	1.0	1.0
- Yes	1.56 (1.02-2.39), p=0.040	1.30 (0.83-2.04), p=0.260

Table 2 Continued.

	Hazard ratio (95% confidence interval)	
	Whole cohort (n=287)	Vulvar radiotherapy excluded (n=236)
Patient- and treatment characteristics		
HSIL in margin		
- No	1.0	1.0
- Yes	0.54(0.20-1.48), p=0.233	0.46(0.15-1.47), p=0.189
Tumor characteristics		
Growth pattern		
- Invasive	1.0	1.0
- Spray	1.60 (0.94-2.70), p=0.081	1.46 (0.82-2.59), p=0.201
- Confluent	1.36 (0.77-2.41), p=0.292	1.34 (0.74-2.44), p=0.336
- Mixed	0.56 (0.08-4.11), p=0.564	0.51 (0.07-3.77), p=0.508
LVSI		
- No	1.0	1.0
- Yes	1.00 (0.58-1.72), p=0.99	0.98 (0.54-1.77), p=0.933
Grade of differentiation		
- Good	1.0	1.0
- Moderately	1.45 (0.88-2.40), p=0.148	1.40 (0.82-2.37), p=0.219
- Poor	1.41 (0.79-2.50), p=0.242	1.45 (0.79-2.68), p=0.234
Tumor diameter (continuous)	1.01 (0.99-1.02) p=0.666	1.00 (0.99-1.02), p=0.865
- < 40 mm	1.0	1.0
- ≥ 40 mm	0.92 (0.55-1.54), p=0.763	0.81 (0.44-1.51), p=0.511
Depth of invasion (continuous)	1.02 (0.97-1.07) p=0.381	1.03 (0.98-1.09) p=0.281

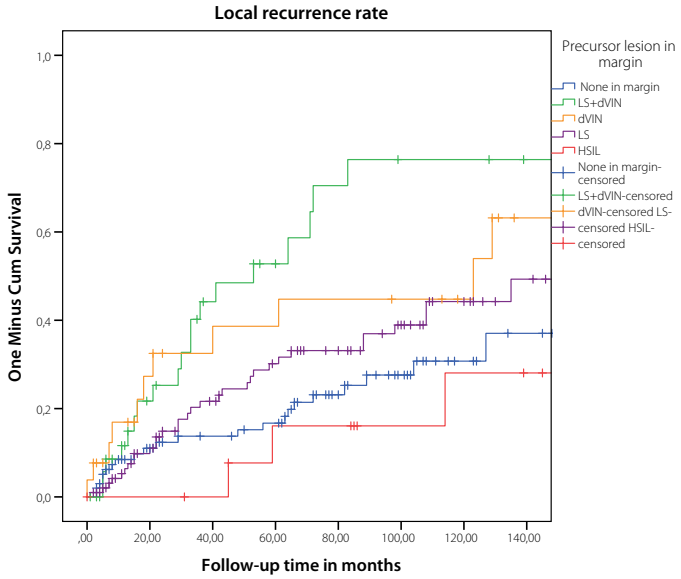
Footnote: HSIL: high-grade squamous intraepithelial lesions , dVIN: differentiated vulvar intraepithelial neoplasia, LVSI: lymph-vascular space invasion.

Tumor characteristics in relation to local recurrences

The growth pattern, presence of lymph-vascular space invasion (LVSI), grade of differentiation, tumor diameter and depth of invasion had no effect on the local recurrence rate, as displayed in Table 2.

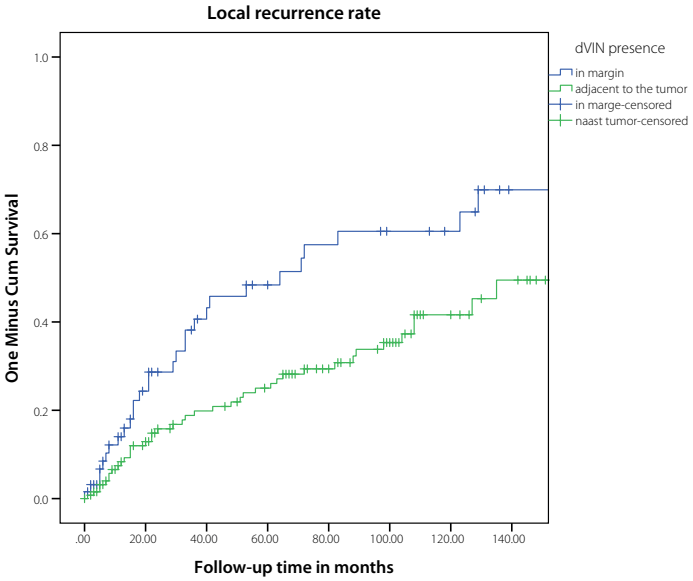
Multivariable analyses

Our multivariable model shows that the presence of dVIN combined with LS or without LS in the margin is associated with a higher local recurrence rate (HR 2.76 (95% CI 1.62-4.71), p < 0.001, HR 2.14 (95% CI 1.11-4.12), p = 0.023). Furthermore, FIGO stage II or higher also resulted in a higher local recurrence rate (HR 1.62 (95% CI 1.05-2.48), p = 0.028), displayed in Supplementary Table 3.



Months	0	24	60	120	144	**Univariable Cox-regression** HR (95% CI)
Number at risk						
- None in margin	103	64	56	14	9	1.0
- LS and dVIN	39	20	9	3	1	3.32 (1.79-6.16), p<0.001
- dVIN	26	12	10	6	1	2.28 (1.10-4.71), p<0.042
- LS	104	66	47	19	9	1.45 (0.84-2.49), p<0.183
- HSIL	15	15	10	6	5	0.83 (0.28-2.41), p<0.728

Figure 2 Local recurrence rate by presence of precursor lesions in margin



Months	0	24	60	120	144
Number at risk					
- dVIN in margin	65	32	19	9	2
- dVIN adjacent to the tumor	132	86	70	22	11

Figure 3 Local recurrence rate in patients with dVIN; presence of dVIN adjacent to the tumor versus dVIN in the margin

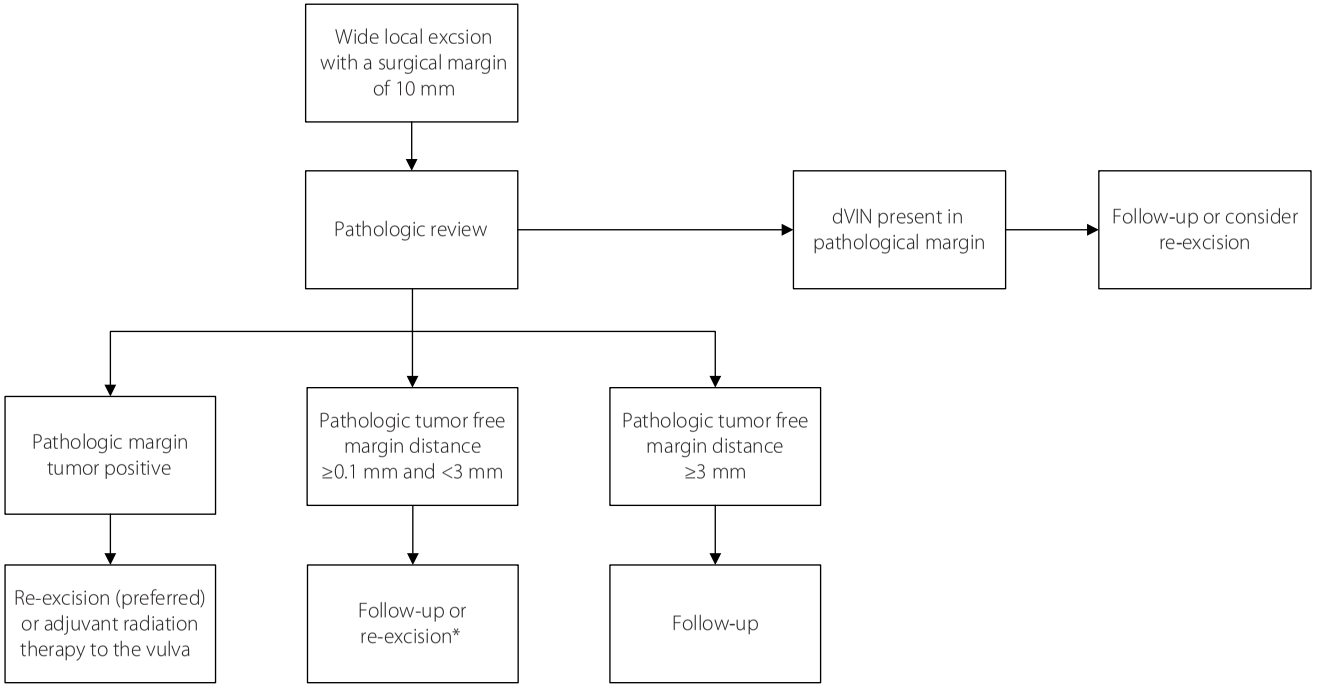
DISCUSSION

In this study, we show that local recurrences are not associated with (any) tumor free margin distance, but strongly with dVIN in the pathologic margin. Our research was carried out on large, clinically well-documented consecutive series of vulvar squamous cell carcinoma patients primarily treated with surgery with expert histopathologic revision. In our study a pathologic tumor free margin distance of ‹ 8, 5 or 3 mm is not associated with a higher local recurrence rate compared to a wider tumor free margin. Most guidelines recommend a surgical tumor free margin of ≥ 15 mm [3] or 10-20 mm [4]. The European Society of Gynecologic Oncology (ESGO) guideline recommends a surgical excision margin of at least 10 mm, while a narrower margin is considered acceptable when the tumor lies close to midline structures (clitoris, urethra, anus) and preservation of their

function is desired [5]. For the ESGO vulvar cancer guideline the current (preliminary and unpublished) data and data from other more recent studies and reviews that questioned the influence of tumor free margin distance were taken into account [2, 6, 7, 15]. Due to a limited amount of patients with a pathologic tumor free margin of < 3 mm in our cohort, we could not determine the prognostic impact of this subgroup. Our data in larger number of patients clearly indicates that pathologic tumor fee margins of ≥ 3 mm do not relate with the local recurrence rate. In our cohort, in which a surgical margin distance of > 10 mm was pursued, approximately 7% of the patients had a tumor positive margin and 8% a tumor free margin between 0 and 3 mm at primary excision. De Hullu *et al.* previously showed that in case of an intended surgical tumor free margin of 10 mm, 50% of the patients have a pathologic tumor free margin of ≤8 mm [16]. This might be due to smaller surgical margin if the tumor was close to important midline structures, shrinkage at fixation, but also because not all of the tumor is macroscopically visible. Therefore, we now recommend an intended surgical tumor free margin of 10 mm, but also not to excise unnecessary tissue close to important midline structures such as the clitoris (see flowchart Figure 4). Future implementation of a smaller tumor free margin will expose less patients to the potential harmful and often mutilating therapies. In selected patients Mohs microsurgery technique, widely applied in patients with skin cancer (eg. in the face), where close but free margins are accepted, might also be useful in vulvar carcinoma cases where small surgical margins are needed because important structures (clitoris, anus) need to be preserved. However currently no data on Mohs and vulvar carcinoma exist.

No differences in local recurrences were found between patients that did or did not receive adjuvant radiotherapy on the vulva (Supplementary Table 1). Therefore, one should be reluctant with adjuvant radiotherapy on the vulva in case of small pathologic margins, since the morbidity of this therapy is high.

Our study shows a significant difference in local recurrence rates related to the presence of precursor lesions of the LS related pathway, which is in line with the study of Yap et al. [9] DVIN in the margin, whether or not in combination with LS, leads to higher local recurrence rates. Therefore, it is important for both the clinician and the pathologist to recognize dVIN. Efforts should be made to explore on how to improve clinical recognition of dVIN by clinician. Recognition of dVIN by the pathologist is of equal importance. Van den Einden *et al.* described histologic characteristics that are most important in the recognition of dVIN [17]. In addition, the authors concluded that it is of added value to revise specimens with an unclear diagnosis and/or clinical suspicion for dVIN by an expert gynaecopathologist. Immunohistochemistry could be helpful in some cases; p16 could be used to exclude HPV-related lesions and p53 might be useful in non-HPV related lesions. However, the exact diagnostic advantage of these and other immunohistochemistry stainings should be further researched to help identify dVIN.



*If re-excision is possible in relation to important structures.

Figure 4 Recommendations for adjuvant therapy after wide local excision of vulvar squamous cell carcinoma

Treatment of precursor lesions should be key in lowering the local recurrence rate. Our data show that patients with both dVIN and LS or dVIN alone in the pathologic margin have significantly higher local recurrence rates. The local recurrence rate 10 years after treatment is as high as 76% in patients with dVIN and LS in the margin compared to 31% for patients with no precursor lesion in the margin ($p < 0.001$, see Figure 2). This might be explained by the concept of field cancerization; the vulva is a field with genetically altered cells with a high risk of developing a precursor lesion and/or carcinoma. Additionally, within the group of patients with dVIN in the resection specimen, patients with dVIN in the pathologic margin suffered significantly more from local recurrences compared to patients with dVIN adjacent to the tumor ($p = 0.002$, Figure 3). Therefore, we recommend to excise lesions suspicious for dVIN during resection of the primary tumor, while re-excision should be considered if dVIN is present in the pathologic margin (see flowchart Figure 4).

Treatment of underlying dermatoses must be one of the major focuses during follow-up. In patients with LS treatment with topical corticosteroids may reduce the risk for developing vulvar carcinoma [2, 18, 19]. Newer treatments such as lipo-injection, ablative laser treatment, and photodynamic therapy have been suggested, but no data are available on whether these therapies also reduce the malignant potential of LS or not. For dVIN, the malignant potential is higher and the time for progression to vulvar carcinoma shorter compared to LS [20-23]. Local (re)excision of dVIN is first choice of treatment. However, clinically it is often difficult to identify the exact location and borders of dVIN. Besides, excision can be mutilating especially when close to functional midline structures. In the future, alternative local treatment regimens such as targeted- or immunotherapy in these patients should be explored.

None of the studied histopathologic characteristics of the tumor were associated with a higher local recurrence rate. This finding is in line with the hypothesis that mostly all local recurrences are 'de novo' tumors arising in a premalignant field. In previously reported studies, authors artificially classified local recurrence in 'de novo' tumors and 'true' local recurrence based on either the location (> or < 2 cm from primary tumor, or ipsilateral or contralateral side of the vulva) or time to local recurrence (< or > 2 years) [1, 6, 9, 24, 25]. In our opinion, both classifications are too arbitrary and currently have no clinical consequences and therefore not used in our study. Future research should be performed on clonal or genetic relationship analyses to distinguish a 'de novo' tumor and a 'true' local recurrence.

In our study cohort, we showed an ongoing risk for local recurrence. The first local recurrence was diagnosed as long as 202 months after primary treatment. No subgroup of patients could be identified with a negligible risk for local recurrence (Figure 2 and Supplementary Figure 2) and there were no differences in median time to local recurrence between the different precursor lesion groups ($p = 0.08$) (See Figure 2 and Supplementary Figure 2). Therefore, we recommend life-long follow-up for all patients

treated for vulvar carcinoma with the aim to identify local recurrences as early as possible, but also to identify and treat precursor lesions to prevent a local recurrence. Oonk *et al.* reported that 65% of the recurrences were detected at routinely scheduled follow-up meetings, indicating the need for patient education regarding the ongoing risk for local recurrence [26]. In addition, identification of high-risk patients may further improve patient empowerment, patient education and may also lead to individualized follow-up schedules. The (early) detection of a local recurrence might be improved by self-examination by the patient and/or her partner, besides the instruction to contact the treating physician at time of any symptoms [26, 27]. Currently there is no literature on the efficacy of self-examination for early detection of a local recurrence in vulvar carcinoma. Our study is the first examining the pathologic margin for both the presence of vulvar carcinoma and precursor lesions in a structured way with revision of all slides by two independent expert gynecopathologists. Furthermore, our study is performed in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) rules. Due to the retrospective character of this study, we were not able to reconstruct the exact location of the local recurrence in all patients. Our study did not had a prospective nature; therefore, we had to deal with missing data. A high number of patients are included with dVIN in the pathological margin without adjuvant treatment, due the lack of a standardized treatment protocol for these patients at that time besides the fact that a part of the dVIN lesions were not detected at initial histopathologic examination. As a result of the small number of patients with a pathologic tumor free margin < 3 mm, we were not able to determine the prognostic impact of this subgroup with small pathologic tumor free margins.

In conclusion, our study shows a high local recurrence rate in patients surgically treated for vulvar carcinoma. No relation was found with pathologic tumor free margin distances. Based on our study we advise to lower the recommended cut-off for a save pathological tumor free margin distance to ≥ 3 mm. We found the local recurrence rate to be especially related to the presence of dVIN (whether or not with LS) and we were unable to identify a subgroup with such a low risk that follow-up could be omitted. Our data reinforce that patients and their doctors need to be aware of the lifelong increased risk for local recurrence after surgical treatment for vulvar carcinoma, especially in patients with dVIN in the margin.

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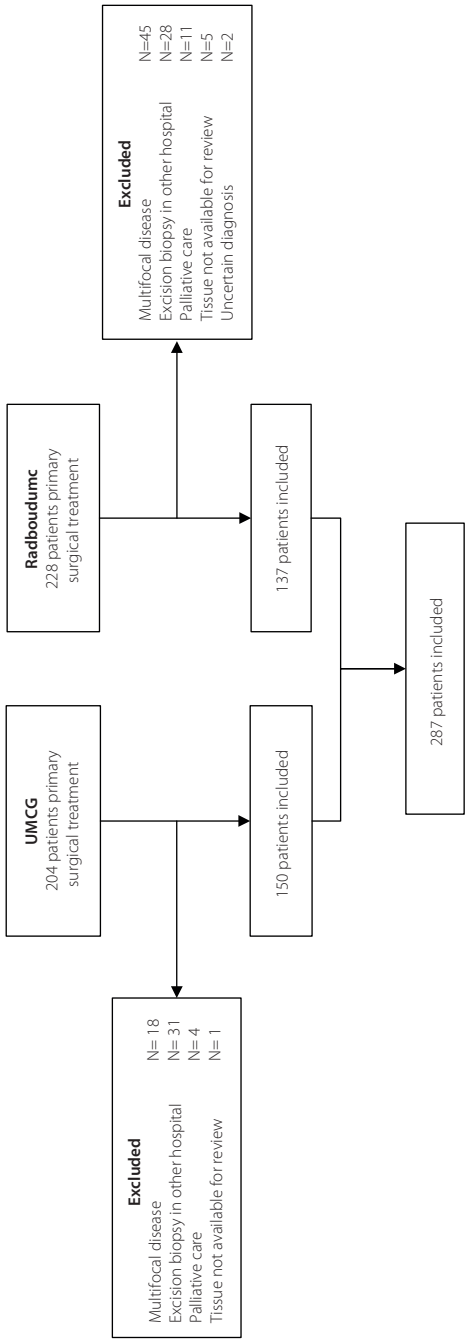
SUPPLEMENTARY DATA



Supplementary Figure 1 Study flow diagram

Supplementary Table 1 Local recurrence rate for different cut-off points for patients without radiotherapy (left part) versus patient that received radiotherapy on the vulva.

	Patients did not receive radiotherapy on the vulva during primary treatment					Patients received radiotherapy on the vulva during primary treatment				
Margin cutoff	Margin < cutoff		Margin ≥ cutoff		Log-rank	Margin < cutoff		Margin ≥ cutoff		Log-rank
	Total N	Local recurrence N (%)	Total N	Local recurrence N (%)		total N	Local recurrence N (%)	total N	Local recurrence N (%)	
3 mm	15	5 (33)	218	70 (32)	p = 0.292	20	4 (20)	29	8 (28)	p = 0.672
5 mm	42	11 (26)	190	65 (34)	p = 0.707	28	7 (25)	21	5 (24)	p = 0.964
8 mm	92	26 (28)	142	50 (35)	p = 0.381	38	10 (26)	11	2 (18)	p = 0.822

N: number

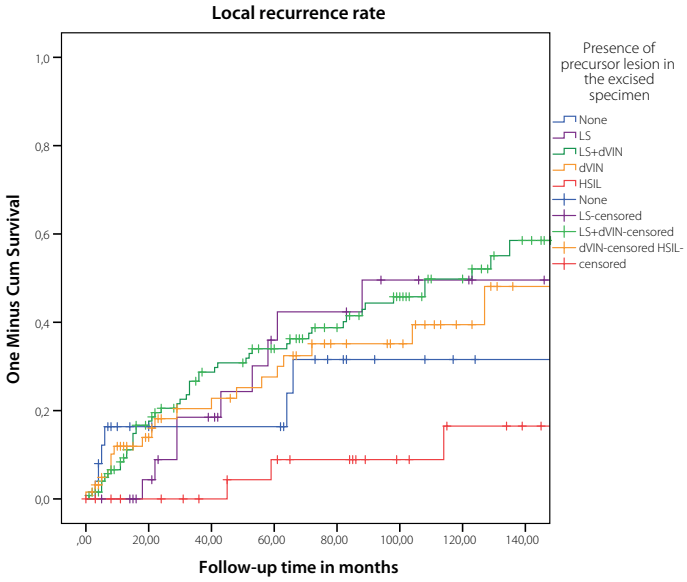
Supplementary Table 2 Presence of precursor lesion in the excised specimen related to local recurrence (univariable).

Presence of precursor lesion in excised specimen	Hazard ratio (95% confidence interval)	
	Whole cohort (n=287)	Vulvar radiotherapy excluded (n=236)
dVIN and LS		
- No	1.0	1.0
- Yes	1.58 (1.04-2.41), p=0.034	1.42 (0.90-2.23), p=0.129
dVIN		
- No	1.0	1.0
- Yes	1.80 (1.08-2.99), p=0.024	1.85 (1.06-3.21), p=0.029
LS		
- No	1.0	1.0
- Yes	1.61 (1.03-2.52), p=0.036	1.39 (0.87-2.22), p=0.172
HSIL		
- No	1.0	1.0
- Yes	0.32 (0.14-0.75), p=0.009	0.27(0.10-0.73), p=0.010

Abbreviations: dVIN: differentiated vulvar intraepithelial neoplasia, LS: lichen sclerosus, HSIL: high-grade squamous intraepithelial lesions

Supplementary Table 3 Multivariable analyses.

Variable	Hazard ratio (95% confidence interval)	p-value
dVIN and LS present in margin	2.76 (1.62-4.71)	p<0.001
dVIN present in margin	2.14 (1.11-4.12)	p=0.023
FIGO stage ≥II	1.62 (1.05-2.48)	P=0.028



Months	0	24	60	120	144	**Univariable Cox-regression** HR (95% CI)
Number at risk						
- None	26	13	13	2	1	1.0
- LS	34	19	10	5	3	1.12 (0.40-3.15), p<0.830
- LS and dVIN	133	81	59	23	10	1.42 (0.61-3.31), p<0.419
- dVIN	64	37	30	8	3	1.14 (0.46-2.86), p<0.776
- HSIL	30	26	20	10	8	0.34 (0.10-1.21), p<0.097

Supplementary Figure 2 Presence of precursor lesions in the excised specimen and the local recurrence rate

Limiting the morbidity of inguinofemoral lymphadenectomy in vulvar cancer patients; a review

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ABSTRACT

Introduction Inguinofemoral lymphadenectomy (IFL) is performed in the treatment for vulvar cancer. One or more complications after IFL is reported in up to 85% of the patients. This review presents an overview of surgical techniques and peri- and post-operative care that has been studied in order to reduce the morbidity associated with IFL in vulvar cancer patients.

Areas covered Current knowledge on postoperative complications after different surgical techniques and peri- and post-operative protocols were discussed. A systematic literature review was conducted using MEDLINE, EMBASE and the Cochrane library on 20 February, 2017. In order to be eligible for inclusion, studies must report the associated post-operative morbidity per surgical technique, or peri- or postoperative care given after IFL in vulvar cancer patients.

Expert commentary After the implementation of several new surgical techniques, the morbidity after IFL decreased but remains high and clinically meaningful. More research is needed on surgical techniques and peri-or postoperative care to further reduce the complication rates after IFL in vulvar cancer patients.

INTRODUCTION

Vulvar cancer is the fourth most common gynecologic cancer after endometrial, ovarian and cervical cancer. Vulvar cancer is a rare disease with an incidence of 2.4 per 100,000 women [1]. Over the past few decades, the incidence of vulvar cancer increased [2]. Vulvar cancer mostly affects elderly women, with more than half of the patients being above the age of 70 years at the time of diagnosis. The most common histological type of vulvar cancer is squamous cell carcinoma (SCC), which accounts for over 80% of the cases [3].

The dissemination of vulvar SCC may occur by three different routes; direct extension, lymphogenic spread and hematogenic spread. The initial spread occurs usually to the inguinofemoral lymph nodes. As soon as the depth of infiltration is more than 1 mm, inguinofemoral lymph node metastases can already be present. Therefore, evaluation of inguinofemoral lymph nodes is crucial. The cornerstone in the treatment of primary vulvar SCC is surgery, consisting of both local tumor resection and inguinofemoral lymph node staging and/or dissection. The treatment for vulvar cancer has developed throughout the last decades. For years, radical vulvectomy with 'en bloc' bilateral inguinofemoral lymphadenectomy was a well established treatment. In the last three decades, less radical surgery was introduced to decrease morbidity with comparable or improved prognosis. Nowadays, the standard treatment of early stage vulvar SCC consists of radical local excision of the tumor combined with a sentinel node (SLN) procedure and/or inguinofemoral lymphadenectomy (IFL). The SLN procedure is safe to perform instead of an IFL in patients with a unifocal tumor <4 cm without suspicious lymph nodes in the groins [4]. In patients with a larger tumor, multifocal disease and/or a positive SLN and in patients with local recurrent disease without earlier IFL, an IFL is indicated. After the implementation of the SLN, in approximately half of the patients with vulvar SCC an IFL is still indicated [5].

Unfortunately, IFL is associated with significant short- and long term complications. The occurrence of one or more groin wound complications is reported in 66-85% of the patients [6,7]. Wound breakdown, wound infection, lymphoceles, lymphedema, cellulitis, and erysipelas are the most reported complications. Because IFL will always have a place in the treatment of vulvar cancer, it is important to look for adjustments, which may reduce the associated morbidity.

The aim of this review is to create an up to date summary of surgical techniques and peri- and postoperative care that have been performed to reduce the morbidity of IFL in vulvar cancer patients.

METHODS

Literature search

We performed a systematic search of the literature on 20 February 2016 using MEDLINE, EMBASE, and the Cochrane library. The following search (MeSH)terms and synonyms were used for 'lymphadenectomy' combined with 'vulvar cancer' (lymphadenectomy, inguinofemoral lymphadenectomy, groin surgery, groin dissection, lymph node dissection, lymph node surgery, lymph node excision; vulvar cancer, vulvar tumor, vulvar carcinoma, vulvar neoplasm, vulvar squamous cell carcinoma). The search was restricted to the English and Dutch language.

Study selection

In order for an article to be eligible, the following predefined criteria had to be met [1]: (randomized) (un)controlled trial; controlled or uncontrolled prospective or retrospective study [2]; IFL for vulvar cancer patients aged >18 years and [3] report the associated complication rate per studied surgical technique or peri- or post-operative care.

Outcome measures

Our primary outcomes of interest were;

- Complication rate per surgical technique and/or peri-, or post-operative care
  - Short-term complications <8 weeks after surgery
    - Wound breakdown
    - Wound infection
    - Lymphocele
  - Long-term complications >8 weeks surgery
    - Cellulitis or erysipelas (recurrent)
    - Lower extremity lymphedema

Secondary outcome of interest was;

- Quality of life

Data extraction and analysis

Relevant data on study population, study design, surgical technique, peri-or postoperative protocol, and the complication rates were extracted from the included studies. Similar complications were grouped together such as seroma and lymphocele, cellulitis and erysipelas, wound dehiscence and wound breakdown.

Quality of the included studies was categorized according to the levels for intervention studies of evidence of the Oxford Centre of Evidence Based Medicine [8]. Level one evidence included a systematic review of randomized controlled trials (RCT), level two, evidence included a randomized trial, level three, a non-RCT, level four, case-series, case control studies or historically controlled studies.

The aim of this review was to create an up-to-date summary of all available evidence regarding surgical techniques and peri- and postoperative care protocols after IFL in vulvar cancer patients. Therefore, we included all published articles concerning this subject. As both controlled and uncontrolled trials were expected to include in this review, we planned not to perform meta-analyses.

RESULTS

Literature search

The search yielded a total of 1988 articles, after removal of duplicates, 1363. One author (AP) screened all titles and checked for relevant abstracts; 123 abstracts were found to be relevant and were retrieved in full text. The same author assessed the articles for eligibility using the predefined criteria as stated in our methods. A total of 36 studies were included in this review.

Study characteristics

The research designs of the included studies were mainly retrospective (23 studies), six were prospective uncontrolled studies, five RCTs and two prospective controlled studies. Study populations ranged between 5 and 194 patients and were studied in the period from 1955 until 2016. The quality of the included studies was mainly level four evidence (31 studies).Two included RCTs were downgraded to level three evidence because they did not perform a sample size calculation and both included less than 15 patients [9, 10] (See table 1).

The following surgical interventions were studied: separate incisions or radical vulvectomy with 'en bloc' lymphadenectomy, unilateral IFL, sparing of saphenous vein, Sartorius transposition, dura mater for femoral vessel coverage, minimally invasive technique, preservation of fascia lata, harmonic scalpel or electrosurgery, plasmajet, VH fibrin sealant, lymphatic microsurgical venous anastomosis (LYMPHA technique), lymphatic flap, method of skin closure, anticoagulation, postoperative drainage and compression garments. None of the included studies reported data on quality of life. For an overview of the association of the above described interventions on post-operative morbidity after IFL, see Table 2.

Modifications of surgery

Type of groin incision

The morbidity of IFL by type of groin incision was reported by ten included studies, both separate incisions and the 'en bloc' approach were described. See Table 3.

Only two retrospective studies studied postoperative complications after IFL by separate incisions versus the 'en bloc' approach [14, 15]. Helm *et al.*[14] did report a

Table 1 Characteristics of included studies

Study	Year	Design	Patients	Intervention	Control	Level of evidence
Hacker 1981 [11]	1957-1978	Retrospective	100	Separate incisions	*	4
Piver 1983 [12]	1957-1971	Retrospective	115	Sodium warafin, dextran, none	Heparine	4
Podratz 1983 [7]	1955-1975	Retrospective	175	'en bloc' approach		4
Fiorica 1991 [13]	1987-1990	Prospective	20	Cadaver dura mater	*	4
Helm 1992 [14]	1969-1988	Retrospective	64	Separate incisions	'en bloc' approach	4
Lin 1992 [15]	1970-1988	Retrospective	82	Separate incisions	'en bloc' approach	4
Finan 1994 [16]	1991-1992	Prospective	11	Artificial dura film	*	4
Burke 1995 [17]	1978-1994	Retrospective	76	Bilateral and/or unilateral IFL	*	4
Paley 1997[18]	1975-1994	Retrospective	101	Sartorius transposition	Not sartorius transposition	4
Bell 2000 [19]	1990-1998	Retrospective	60	Preservation of the fascia lata	*	4
Zhang 2000 [20]	1990-1998	Retrospective	83	Preservation saphenous vein	Ligation saphenous vein	4
Gould 2001 [21]	1992-1999	Retrospective	67	Seperatie incisions	*	4
Gori 2002 [22]	1992-1997	Retrospective	45	Unilateral IFL	Bilateral IFL	4
Gaarenstroom 2003 [6]	1993-2000	Retrospective	101	Separate incisions	*	4
Rouzier 2003 [23]	1978-2000	Retrospective	194	Sartorius transposition	Not sartorius transposition	4
				Preservation of saphenous vein	Ligation saphenous vein	
Judson 2004 [24]	1996-2002	RCT	61	Sartorius transposition	Not Sartorius transposition	2
Micheletti 2005 [25]	1981-2002	Retrospective	156	Preservation fascia lata	*	4
Dardarian 2006 [26]	1992-2003	Retrospective	29	Sparing saphenous vein	Ligation saphenous vein	4
Zhang 2007 [27]	1989-2005	Retrospective	64	Preservation saphenous vein	Ligation saphenous vein	4
Carlson 2008 [28]	2002-2005	RCT	137	VH fibrin sealant	No VH sealant	2

Pellegrino 2008 [29]	2005-2007	Retrospective	42	Harmonic scalpel	Conventional electrosurgery	4
Van der Zee 2008 [4]	2000-2006	Prospective	457	Separate incisions	*	4
Manci 2009 [30]	2000-2007	RCT	62	Inferior skin incision	Superior skin incision	2
Sawan 2009 [10]	2006	RCT	14	Prophylactic compression garments	Not prophylactic compression garments	3
Hinten 2011 [5]	1988-2009	Retrospective	164	'en bloc' approach and separate incisions	*	4
Madhuri 2011 † [9]	Not reported	RCT	18	Plasmajet	Not plasmajet	3
Walker 2011 [31]	2001-2009	Retrospective	50	Continuous subcuticular suture	Staples	4
			56	Separate incisions Length of drainage	* *	
Xu 2011 [32]	2008-2010	Retrospective	17	VEIL abdominal approach	*	4
Li 2012 [33]	2004-2009	Retrospective	24	Modified triple incisions	*	4
Soliman 2012 [34]	2002-2009	Retrospective	34	Separate incisions	*	4
Morotti 2013 [35]	2009-2011	Prospective	15	LYMPHA	No LYMPHA (historical cohort)	4
Li 2015 [36]	2007-2013	Prospective	58	Sartorius tendon transposition	Sartorius transposition	4
Wang 2015 [37]	2010-2013	Prospective	21	VEIL hypogastric subcutaneous approach	*	4
Gentileschi 2016 [38]	Not reported	Prospective	5	Lymphatic flap	No lymphatic flap	4
Wu 2016 [39]	2011-2016	Prospective	37	VEIL lateral approach	*	4
Jain 2017 [40]	2011-2015	Retrospective	12	R-VEIL	*	4

Footnotes: †- conference abstract , *-not studied, NR: not reported, IFL: inguinofemoral lymphadenectomy, VEIL: video endoscopic lymphadenectomy, LYMPHA: lymphatic microsurgical preventive healing approach, R-VEIL: robot-assisted video endoscopic inguinal lymphadenectomy.

Table 2 Influence of intervention on the complication rate after IFL per level of evidence

Level of evidence	Decrease	Increase	No association	Unclear
Level 1	None	None	None	None
Level 2	None	Fibrin sealant	Sartorius transposition Skin access above or below the inguinal ligament	None
Level 3	None	None	None	Plasmajet Prophylactic compression garment
Level 4	Separate incisions Unilateral IFL Sparing saphenous vein Preservation fascia lata LYMPHA* Continuous suture	'en bloc' approach Bilateral IFL Artificial dura mater	Cadaver dura mater	(R-)VEIL Harmonic scalpel or electrosurgery Anticoagulation Duration of drainage Lymphatic flap

Footnotes: *: only lymphedema studied, (R-) VEIL: (robot assisted) video endoscopic inguinofemoral lymphadenectomy, IFL: inguinofemoral lymphadenectomy, LYMPHA: lymphatic microsurgical preventive healing approach

reduction of patients with wound breakdown using separate incisions but this reduction was not statistically significant. The second study described a reduction of the wound breakdown rate and an increase in lymphoceles after using separate incisions, but did not perform any statistical tests [15]. Six other studies report the incidence of postoperative complications after the use of separate incisions without a control group. Overall, it can be concluded that the occurrence of wound breakdown, lymphedema and cellulitis/erysipelas was reduced by using separate incisions with an increase in lymphoceles.

To determine further reduction of the morbidity associated by IFL using separate incisions, Manci *et al.* [30] compared skin access above or below the inguinal ligament in a RCT and found an advantage for incision above the ligament; wound dehiscence in 32% versus 17%, lymphocele in 19% versus 6% respectively, although there was no statistically significant difference.

Laterality of IFL

Unilateral IFL as an alternative to bilateral IFL was studied in a selected group of patients with lateralized tumors (tumor with medial margin >1 cm from the midline) without palpable groin lymph nodes. Two studies reported less complications in unilaterally treated patients [17, 22]. The number of patients with wound dehiscence or

lymphedema is significantly reduced by performing a unilateral IFL from 24% to 0% and 67% to 8% respectively ($p < 0.001$)[22]. In conclusion, unilateral IFL does reduce morbidity but can only safely be performed in patients with early stage and lateralized vulvar cancer [17].

Sparing of the saphenous vein

The classic description of IFL includes ligation of the saphenous vein. It was suggested that sparing the saphenous vein might reduce the complications associated with IFL. Four retrospective studies reported the complication rates after sparing versus ligation of the saphenous vein [20, 23, 26, 27]. See table 4 for an overview of the outcomes of these studies. There was a significant lower number of groins with wound infection [23, 26], wound breakdown [20, 23, 26], lymphedema [23, 26, 27] and cellulitis/erysipelas [20, 27] after sparing of the saphenous vein. There was no evidence of effect for the reduction of lymphoceles. It can be concluded that sparing of the saphenous vein reduces the postoperative wound complications after IFL.

Coverage of femoral vessels

The transposition of the Sartorius was first introduced by Way as modification to protect the femoral vessels in case of wound breakdown and to decrease the morbidity after IFL [41]. The effect of transposition of the Sartorius on the complication rate is reported in three studies, one RCT [24] and two retrospective studies [18, 23]. Judson *et al.* [24] randomized patients for either Sartorius transposition or not. They found a statistically significant increase of lymphocele formation in patients in the transposition group. Other outcomes, such as wound breakdown, wound infection and lymphedema did not differ between those two groups of patients.

One retrospective study reported an increase in patients with lymphedema after transposition of the sartorius, with statistical significance [23] and another study reported a reduction of wound infections after transposition [18]. These studies did not report significant differences between wound breakdown and lymphocele. See Table 5 for an overview of results.

Another explored surgical technique during IFL to cover the groin vessels is Sartorius tendon transposition versus Sartorius transposition as investigated by one case-control study [36]. Wound breakdown and chronic lymphedema were significantly reduced in the Sartorius tendon transposition group, 30% versus 3.6% ($p = 0.012$) and 33.3 versus 7.1% respectively ($p = 0.022$). However, none of the studies report the morbidity after tendon transposition versus no tendon or Sartorius transposition.

In the past, cadaver dura mater was used to cover the groin vessels but this is difficult and more time consuming than Sartorius transposition, besides the risk of transmittance of viral infections [13]. The artificial dura mater was not effective and increased the complications after IFL [16].

Table 3 Separate incisions versus 'en block' resection for inguinofemoral lymphadenectomy; complications in percentages

Study	N	Short term									Long term						Overall ≥1 complication
		Wound infection			Wound breakdown			Lymphocele			Lymphedema			Cellulitis/erysipelas			
		Separate	En bloc	P-value	Separate	En bloc	P-value	Separate	En bloc	P-value	Separate	En bloc	P-value	Separate	En bloc	P-value	
'en bloc' versus separate incisions per patient																	
Helm 1992	64	-	-		19	34	NS	31	19	NS	-	-		22	16	NS	-
Lin 1992	82	-	-		22	53	-	15	0	-	15	13	-	-	-	-	-
'en bloc' per patient																	
Podratz 1983	175		85†			85†			11			69			13		-
Separate incisions per patient																	
Gaarenstroom 2003	101	39			17			40			28				-		66
Gould 2001	67	35			19			13			30				22		-
Hacker 1981	100	9			44			13			20				2		-
Hinten 2011††	164	29			19			29			49				34		-
Van der Zee 2008 ‡	166	21			34			-			25				16		-
Separate incisions per groin																	
Gaarenstroom 2003	187	27			11			27			21				-		52
Soliman 2012	64	3			10			13			5				24		-

†; including wound infection, wound breakdown and necrosis, ††both en bloc and separate incisions, ‡sentinel node procedure with subsequent inguinofemoral lymphadenectomy

In conclusion, there is no evidence that covering the femoral vessels by transposition of the Sartorius or cadaver or artificial dura mater does reduce the complications after IFL in vulvar cancer patients.

Minimally invasive IFL

The last years, minimally invasive techniques were developed for IFL to reduce the postoperative complications. After the introduction in other malignancies such as penile cancer and melanoma, video endoscopic IFL (VEIL) was studied in vulvar cancer patients. Until now, there is limited English literature on vulvar cancer. Four studies concerning VEIL in vulvar cancer patients were included in this review [32, 37, 39, 40]. These studies were published between 2011 and 2017, two were prospective and two retrospective. In total, 87 patients were included (75 patients from China and 12 patients from India). For results see Table 6.

Xu *et al.* used the abdominal approach for VEIL and reported no inguinal wound-related complications besides one patient with exhibited lymphorrhea through the drain orifice [32]. The second study used a hypogastric subcutaneous approach and reported only one patient with a lymphocele (5%); furthermore no other inguinal wound complications were observed [37]. The third study used a 3-incision lateral approach to perform VEIL in 37 patients and was the largest published study in vulvar cancer patients. They described wound breakdown in 3% of the patients; no other complications such as wound infection, lymphocele, lymphedema and cellulitis were reported in these patients [39]. The last study performed in India used robot-assisted VEIL (R-VEIL) to perform IFL. Reported overall complication rate was 75% per patient and 60% per groin [40]. These overall complication rates reported after R-VEIL were even higher to those reported after open IFL by Gaarenstroom *et al.* of 66% per patient and 52% per groin respectively [6]. Furthermore, it is unclear if (R-)VEIL is safe in vulvar cancer patients, given a groin recurrence rate as high as 6% in patients using VEIL [39] comparing to 2.5% after using the conventional procedure [42].

In conclusion, (R-)VEIL seems to be feasible for the approach of IFL as described in all studies, but it remains questionable if (R-)VEIL reduces the postoperative complications, and whether this procedure is oncologically safe or not.

Other modifications

More individual studies report modifications on the current surgical techniques of IFL. First of all, the preservation of the fascia lata. Two retrospective studies report after sparing of the fascia lata lower rates of lymphocele, lymphedema, wound infection, and wound breakdown compared to the literature[19, 25] and overall short-term complication rate of 38% and long term complication rate of 14% [25].

The device used during IFL is studied as a possible option to reduce the morbidity. Pellegrino *et al.* [29] investigated the differences in postoperative complications of using

either the harmonic scalpel or conventional electrosurgery. They did not report significant differences between these two groups in the incidence of postoperative complication. In this study only one patient had a postoperative complication in the harmonic scalpel group (including 22 patients) versus zero patients in the conventional electrosurgery group (including 20 patients). Another study regarding the surgical device used is performed by Madhuri *et al.* [9] using Plasmajet (a surgery system utilizing kinetic energy and highly controlled thermal effects to seal the tissue). In a conference abstract they described a pilot RCT in 18 patients and concluded that Plasmajet may reduce the lymphocele formation but did not publish the full paper until now.

Another studied option is the sealing of lymph vessels during IFL, evaluated by Carlson *et al.* [28] in a RCT. They investigated the addition of VH fibrin sealant sprayed in the groin before sutured closure. Inguinal infections, wound breakdown, lymphoceles and lymphedema did not significantly differ between the two groups. However, they reported a significant increase in the number of vulvar infections in patients treated by VH fibrin sealant (33% versus 14%, p = 0.0098).The overall complication rate for patients treated in the intervention group was 61% versus 59% in the control group. In conclusion, VH fibrin sealant in the groin did not reduce the groin complications after IFL and increased the vulvar wound complications.

The use of lymphatic microsurgical preventive healing approach (LYMPHA) in the prevention of lymphedema during IFL was described by one prospective study in 23 groins [35]. Before incision of the skin, blue dye was injected in the thigh muscles to identify the lymphatic vessels; anatomizes were made between lymphatics from the lower limb and one of the collateral branches of the femoral vein. They reported lymphedema in 8% of the groins after the LYMPHA technique and 36% in the cohort without LYMPHA technique after a mean follow up of 16.7 (SD 6.2) months. Other groin wound complications were not reported. More research is needed before implementation of this technique. Another study used a surgical technique for microsurgical reconstruction of the lymphatic drainage in order to prevent lymphatic drainage impairment. In this study, a lymphatic flap harvested from the flank was used to create a bridge in the gap of lymphatic vessels created by IFL. This technique was used in one groin, while the conventional surgical method was used for the other groin. Patients were evaluated 6 months after surgery, and the limbs treated by the lymphatic flap showed mild edema versus moderate to severe edema in the untreated limb [38].

The method of skin closure was studied by one retrospective study [31]: continuous subcuticular suture was compared to staples. Lymphocele formation was reduced (21% versus 47%, p = 0.05) and also a reduction in chronic lymphedema (6% versus 29% p = 0.02) by using subcuticular suture compared to staples. Wound infection and wound breakdown were not influenced by the method of skin closure.

Modifications of perioperative care

In 1983, one study reported the influence of perioperative prophylactic anticoagulation as a possible cause of lymphocele after IFL. In patients receiving heparin, 42% developed a lymphocele versus 2% in patients receiving sodium warfarin, dextran or no anticoagulation ($p < 0.01$).The influence of both treatment regimens on the risk of embolism in these patients is unclear. There are no other studies regarding prophylactic anticoagulation and the associated risk of complications after IFL besides this dated publication. Nowadays, the use of heparin is more and more replaced by the use of low-molecular-weight heparin during surgery and postoperative care. No reports about usage of low-molecular-weight heparin are published until now.

The usage of prophylactic intravenous antibiotics is widely accepted during perioperative care, but there were no publications about reducing postoperative (wound) complications.

Modifications of post-operative care

The postoperative care given might influence the morbidity after IFL. First of all, the postoperative drainage of the groin after IFL might reduce the morbidity. The optimal duration of lymph fluid drainage was studied by only two retrospective studies. Walker *et al.* [31] reported complications in association with the duration of lymph fluid drainage. The main reason for removal of the drain was production of < 50ml per 24 hours. Wound breakdown and lymphedema were significantly lower in the group of patients drained over 7 days compared to shorter drainage. There was no influence on the number of patients with a wound infection and lymphocele. Another retrospective study showed that higher drain production on the last day in situ was associated with an increased risk for any short term complication but there was no effect found on duration drain in situ on the complication rate[5]. Prospective research is needed to determine the optimal duration of postoperative drainage in terms of reduction of complications after IFL.

The second studied modification of postoperative care was the use of prophylactic compression garments by Sawan *et al.* [10] in 14 patients. Patients were randomly assigned for prophylactic compression garments or best supportive care without compression garments. There was no statistically significant difference in postoperative complication rate regarding wound breakdown, wound infection, lymphocele and lymphedema. Further (larger) studies are needed to investigate the role of compression garments for reducing the morbidity of IFL.

Table 4 Sparing versus ligation of the saphenous vein; complications in percentages

Study	N	Short term									Long term						Overall ≥1 complication
		Wound infection			Wound breakdown			Lymphocele			Lymphedema			Cellulitis/erysipelas			
		Sparing	Ligation	P-value	Sparing	Ligation	P-value	Sparing	Ligation	P-value	Sparing	Ligation	P-value	Sparing	Ligation	P-value	
Per groin																	
Dardarian 2006	49	0	45	<0.001	0	25	<0.02	0	0	-	11	39	<0.05	0	6	NS	-
Rouzier 2003	355	18	30	0.01	16	36	<0.001	-	-		23	45	<0.001	-	-		-
Zhang 2000	139	-	-		13	38	0.001	10	4	NS	32	70	-	18	39	0.006	-
Zhang 2007	128	68	73	NS	-	-		26	32	NS	25	48	<0.01	21	41	<0.05	-

Table 5 Transposition of Sartorius versus not, complications in percentages

Study	N	Short term									Long term						Overall ≥1 complication
		Wound infection			Wound breakdown			Lymphocele			Lymphedema			Cellulitis/erysipelas			
		Transposition	Not	P-value	Transposition	Not	P-value	Transposition	Not	P-value	Transposition	Not	P-value	Transposition	not	P-value	
Per patient																	
Judson 2004	61	29	30	-	11	9	-	40	15	0.04	18	33	NS	-	-		-
Paley 1997	101	30	58	0.01				16	18	-	24	26	-				-
Per groin																	
Rouzier 2003	206	28	30	NS	43	36	NS	-	-		55	22	<0.001	-	-		-

Table 6 Minimally invasive approach for IFL, complications in percentages

Study	N	Short term			Long term		
		Wound infection	Wound breakdown	Lymphocele	Lymphedema	Cellulitis/ erysipelas	Overall ≥1 complication
Per patient							
Xu 2011	17	0	0	0	0	0	0
Wang 2015	21	-	-	5	-	-	-
Wu 2016	37	-	3	0	-	0	-
Jain 2017	12	0	0	33	33	17	75‡
Per groin							
Jain 2017	22	0	0	27	27	9	60‡

‡: including prolonged lymphhorrhea, - not reported

EXPERT COMMENTARY

Where feasible we advise the implementation of separate incisions, unilateral IFL, sparing of the saphenous vein, preservation of the fascia lata and continuous suture as the standard care for IFL in vulvar cancer patients. These surgical techniques appear to reduce the complication rates. The usage of peri-operative fibrin sealant, the 'en bloc' approach, bilateral IFL (for lateralized tumors), and artificial dura mater should not be used as a standard surgical technique for IFL given the higher complication rates. Sartorius transposition, skin access above or below the inguinal ligament, and cadaver dura mater did not influence the complication rate and should not be implemented for IFL. Minimally invasive approach for IFL is promising in terms of reduced of complication rates, but the oncological safety remains unclear. Therefore, minimally invasive techniques for IFL should only be used within the protection of a prospective trial.

Regarding the postoperative care, there is no consensus about optimal drain management and it remains unclear if prophylactic compression garments do reduce the postoperative complication rates. Peri- and postoperative protocols should be studied more extensively before the implementation of new protocols.

Concerning the surgical technique in vulvar cancer patients, the implementation of separate incisions decreased the morbidity after IFL. Although, a randomized controlled trial comparing the 'en bloc' approach versus separate incisions investigating the oncologic safety is lacking. However, in a Cochrane review all observational studies

concerning separate incisions were pooled and they concluded that separate incisions were deemed to be oncologically safe [43].

Another surgical technique which appears promising in terms of reduction of post-operative morbidity is minimally invasive IFL. Recently, two reviews were published on this subject [44, 45]. One review included ten studies including 236 procedures in 168 patients with penile, vulvar or vaginal cancer or melanoma. They concluded that the number of wound infections, wound dehiscences and lymphoceles appeared lower in patients operated by the VEIL technique (overall complication rate 4% of the groins) compared to the open technique [44]. The other systematic review reported an overall complication rate of 13% (lymphocele formation 3.6%, wound infection 1.2%, and lymphedema 0.4%) [45]. This review included nine studies, but only three out of these were published in English. One was included in our review [32], the others were excluded for this review (one study included also vaginal cancer patients and the other did not report data about postoperative complications). So far, no RCTs assessed this new minimally invasive surgical technique. Furthermore, none of the studies included Caucasian women. Moreover, the usage of an ultracision scalpel, which has proven to reduce the incidence of lymphoceles after axillary dissection in breast cancer patients [46], which is used during VEIL and not during the open procedure may partly explain the differences in complication rates. The results on decreasing the incidence of postoperative complications by using the minimally invasive approach are promising, but the oncologic safety of this technique is still unclear. The main concern is the high groin recurrence rate of 6.5% in patients with negative lymph nodes after VEIL [39]. The oncologic safety of pelvic lymph node dissection by laparoscopy is proven for other gynecological malignancies, no differences in number of lymph nodes removed, disease recurrence and survival were reported in several studies [47, 48]. A large prospective trial with adequate follow up of at least 2 years is needed to determine the oncologic safety of VEIL and the postoperative outcomes in Caucasian women with vulvar cancer.

IFL is also performed in patients with a melanoma or penis cancer. In order to reduce bias, in our review only studies including patients with vulva cancer were included. There were several reasons for excluding other indications for IFL than vulvar cancer. First of all, the median age of patients with melanoma is lower than in patients with vulvar cancer, older patients are faced with more comorbidity than younger patients which plays a role in the incidence of postoperative complications. Furthermore, the sex of a patient may influence the complication rate after IFL; therefore studies on penis cancer, including only men, were excluded from this review. However, more studies on the complications after IFL for melanoma and after axillary dissection for breast cancer are published. Regarding the complications after IFL in melanoma patients, a review included seven studies described different surgical approaches for IFL; applying fibrin sealant in the wound bed, different incision techniques for separate

incisions, local versus general anesthesia, early mobilization or postoperative bed rest were studied, but none of these interventions showed a statistically significant reduction in complication rates [49]. A review concerning seroma formation after axillary dissection in breast cancer patients reported a decrease in complications by using ultrasonic scissors, electrotermal bipolar vessel system, suture fixation techniques to reduce the dead space, volume controlled suction drainage and active shoulder exercise[50].

There are some limitations to this review. First of all, there is a lack of published studies with level one evidence. As vulvar cancer is a rare disease, it is hard and not realistic to perform a large number of RCTs in vulvar cancer patients. Therefore, this review was mainly based on data from retrospective or observational studies, which is a source of bias. Another limitation was the different definitions used for complications after IFL. An attempt should be made to standardize the definitions used for postoperative wound complications in vulvar cancer patients for this will improve the comparability between published studies in the future.

FIVE YEAR VIEW

Even though new surgical modifications were implemented, the complication rate after IFL in vulvar cancer patients remains high. In the near future, more research is expected to further reduce this morbidity. Besides research on reducing the complications after IFL, research will focus on new treatment regimens in order to limit the indication for IFL. Reducing the number of patients with an indication for IFL can be achieved by the introduction of a repeat SLN procedure and/or radiotherapy on the groin. However, it should be kept in mind that the consequences of a groin recurrence are significant, with a 5-year survival rate of only 0-15% [51-53]. Therefore, determination of the lymph node status remains an important part in the treatment of vulvar cancer patients.

The safety of a SLN procedure in patients with a local recurrence of vulvar SCC has not been proven yet [54]. As a consequence, patients with a local recurrence after an earlier negative SLN, do not perceive the benefits of the SLN procedure in terms of the omission of an IFL. Research concerning the safety of a (repeat) SLN procedure instead of an IFL in patients with recurrent vulvar SCC is expected within the upcoming years.

In 2018, results will be expected of the GROINSS-VII study that will provide more information on the safety and efficacy of radiotherapy instead of IFL in patients with micro-metastases in the sentinel lymph nodes.

Replacement of IFL by other treatment options with less treatment related morbidity but the same effectiveness and safety will be a step forward in treating vulvar SCC patients. For patients who still have an indication for IFL, research is expected on minimally invasive techniques for IFL to determine the oncologic safety and the

benefits in terms of reduced postoperative morbidity. Furthermore, the device used during surgery may be a key factor in preventing postoperative complications. Peri- and postoperative care may also play an important role and also affect the postoperative complication rates. More prospective studies regarding the peri-and postoperative care should be performed.

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Volume-controlled versus short drainage after inguinofemoral lymphadenectomy in vulvar cancer patients: A Dutch nationwide prospective study

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ABSTRACT

Objective Inguinofemoral lymphadenectomy for patients with vulvar squamous cell carcinoma is associated with a high incidence of postoperative wound complications, which may be influenced by inguinal drain management. The aim of this nationwide prospective study (MAMBO: Morbidity And Measurement of the BOdy) was to assess the feasibility and the incidence of complications after volume-controlled versus short drainage.

Methods The MAMBO study consisted of two observational studies in all eight oncology centers in the Netherlands, conducted between 2012 and 2016. In the first study, the drain was removed when the production was <30 ml/24 h, except in the first 48 h, and after a maximum of 28 days (MAMBO-IA). In the second study, the drain was removed five days postoperatively regardless of production (MAMBO-IB). We assessed the complications within eight weeks after surgery using logistic regression to compare the incidence of one or more complications between the two drainage protocols, adjusting for possible confounders.

Results We included 77 patients (139 groins) for volume-controlled drainage and 64 patients (112 groins) for short drainage. Volume-controlled drainage was associated with significant less lymphocele formation. Moreover, we found no difference in wound infection or primary wound breakdown. The estimated incidence of one or more complications was 46% per groin after volume-controlled drainage versus 75% after short drainage, (RD 29% (95% CI 8, 49) p = 0.006).

Conclusions This prospective study shows that volume-controlled drainage is associated with significantly less complications compared to short drainage. We therefore recommend volume-controlled drainage after inguinofemoral lymphadenectomy in patients with vulvar squamous cell carcinoma.

INTRODUCTION

Vulvar squamous cell carcinoma (SCC) is a rare disease and accounts for approximately 3-5% of all female genital malignancies [1]. The incidence is approximately 1-2 per 100.000[2]. The standard treatment for patients with early stage SCC of the vulva consists of wide local excision (WLE) of the tumor combined with a sentinel lymph node (SLN) procedure and/or inguinofemoral lymphadenectomy (IFL). In patients with a primary unifocal vulvar SCC measuring less than four centimeters without suspicious groin lymph nodes; a SLN procedure is indicated and an IFL can be safely omitted [3]. Although the SLN procedure is the preferred treatment in the Dutch guidelines, in around half of the patients an IFL is still indicated based on multifocal tumors, tumors larger than four centimeters, recurrent disease and/or positive SLNs [4].

Unfortunately, IFL has significant short- and long-term complications. The most common short-term complications are wound breakdown, wound infection and formation of lymphoceles and are reported in up to 85% of the patients [5]. Development of lymphedema and cellulitis/erysipelas are the most documented long-term complications.

Efforts have been made to adjust the surgical technique for IFL in order to reduce the associated complications. After the implementation of several new surgical techniques such as triple incisions and sparing of the saphenous vein, the morbidity after IFL decreased, but still remains high and clinically relevant [6-9].

The direct postoperative management for patients with vulvar SCC may reduce the incidence of complications; however, it has not been described extensively and only retrospective studies are published regarding this subject. The usage of prophylactic antibiotics or prophylactic compression stockings did not reduce the postoperative complications [10-12]. Regarding postoperative drain management of the groin in vulvar SCC patients, there is no (inter)national consensus on duration of drainage.

Until now, both volume-controlled and short drainage of the groin are used. Published literature used volume-controlled drainage and removed the drain when the output was < 30-50 ml/24 h [6, 10, 13]. However, the reasons for these specific drain policies were not elaborated and none of the studies compared different drain protocols. Removal of the drain irrespective of the amount of output after several days, may be accompanied by an increase in the incidence of lymphoceles [4]. On the other hand, long drainage may increase the risk for infection, prolonged hospital stay and gives more discomfort for the patient.

Therefore we conducted a nationwide prospective study (MAMBO: Morbidity And Measurement of the BOdy) to start with a volume-controlled drainage protocol to assess the feasibility of such a protocol and to assess the incidence of complications; this was followed by a short drainage protocol to finally compare complication rates of both protocols in order to formulate a standardized national drain protocol.

METHODS

In this prospective multicenter MAMBO (MAMBO: Morbidity And Measurement of the BOdy) study patients were included from eight oncology centers which are united in the Dutch Gynaecologic Oncology Group (Radboud university medical center Nijmegen, University Medical Centre Groningen, Center for Gynaecologic Oncology Amsterdam, location The Netherlands Cancer Institute/Antoni van Leeuwenhoek hospital and Academic Medical Centre Amsterdam, Leiden University Medical Centre, Erasmus Medical Centre Rotterdam, Maastricht University Medical Centre and Catharina Hospital, Eindhoven). All participating centers are expert centers in the treatment of vulvar cancer.

Patients aged ≥18 years with a primary or recurrent SCC of the vulva with an indication for unilateral or bilateral IFL were eligible to participate in this study. Patients with an indication for a SLN procedure only, were not eligible for this study. Further exclusion criteria were previous radiotherapy on the vulva or groins, previous pelvic lymphadenectomy, histology other than SCC and an indication for IFL with the 'en bloc' approach of the vulva and groins.

Two consecutive protocols were performed to assess the feasibility of the two drainage protocols and to compare the incidence of complications. First, patients were included in the volume-controlled drainage protocol (MAMBO-IA) during a period of 21 months (October 2012-June 2014). Thereafter the treatment protocol was changed and patients were included in the short drainage protocol (MAMBO-IB) (March 2015-December 2016). There were several reasons why the MAMBO study group has decided not to perform a randomized trial but to choose for two study protocols. First of all, the incidence of vulvar cancer is low. Secondly, there is no standard treatment regimen defined for the duration of drainage. Furthermore, the feasibility of especially the long drainage protocol (patients were discharged from the hospital with drains in situ) was unknown. Both the design and the treatment protocol of both studies were identical except the timing of removal of the drain.

Patients were asked to participate in the MAMBO study during their initial visit at the outpatient clinic. The patient information form was given and all patients had to sign for informed consent for collecting data. The local ethical committees approved both protocols (registration numbers 2012-246 and 2014-1491).

The surgical technique of the IFL consisted of separate incisions parallel to the inguinal ligament. The extent of the dissection was the inguinal ligament cephalad, the adductor longus muscle medially and the sartorius muscle infero-laterally. After opening the cribriform fascia, all nodes bearing fatty tissue medial from the femoral vein was removed as well. No standard ligation of the saphenous vein nor standard sartorius transposition was performed. Thereafter, a high vacuum Redon continuous suction drain was placed in the operated groin. Postoperatively, the drain production

was measured daily and the groin wounds and drains were inspected by a physician during hospital stay and/or by a homecare nurse or general practitioner in discharged patients.

For patients in the volume-controlled drainage group, the drain was removed when the production of the drain was ‹ 30 ml/24 h (except for the first 48 h after surgery) with a maximum of 28 days. For patients treated by short drainage, the drain was removed on the fifth postoperative day (day of operation is defined as day zero) irrespective of the drain production.

The total follow up time was eight weeks after surgery: two and eight weeks postoperatively all patients were seen routinely at the outpatient clinic for a visit and were examined by a gynecologic oncologist. All short-term complications were assessed and comprised wound infection (purulent exudates and/or positive culture and/or erythema, edema and localized pain), primary wound dehiscence (every spontaneous disrupted groin wound ›2 cm) and the occurrence of a lymphocele (collection of lymph fluid in the groin› 5 cm). For a summary of the study protocol see Figure 1.

Statistical analysis

Continuous variables were summarized using the median and range, discrete variables were described by frequencies. We performed both intention-to-treat (ITT) and per-protocol (PP) analyses to assess the incidence of complications and the differences between volume-controlled and short drainage. The ITT analysis was the primary analysis.

To estimate the incidence of wound infection, primary wound dehiscence and lymphocele, we used a generalized linear model with a logit link and a binomial distribution. In order to quantify the differences between volume-controlled and short drainage, risk differences were estimated using this model; and for analysis per groin, a random effect was added for patient. In addition, to compare the incidence of ≥1 complication per patient and per groin between both drainage protocols, we adjusted for the following patient and treatment characteristics: diabetes mellitus, active smoker, number of lymph nodes removed, IFL and vulva excision on the same day, closure technique and ligation of the saphenous vein. Statistical analyses were performed using SPSS version 22.0 [14].

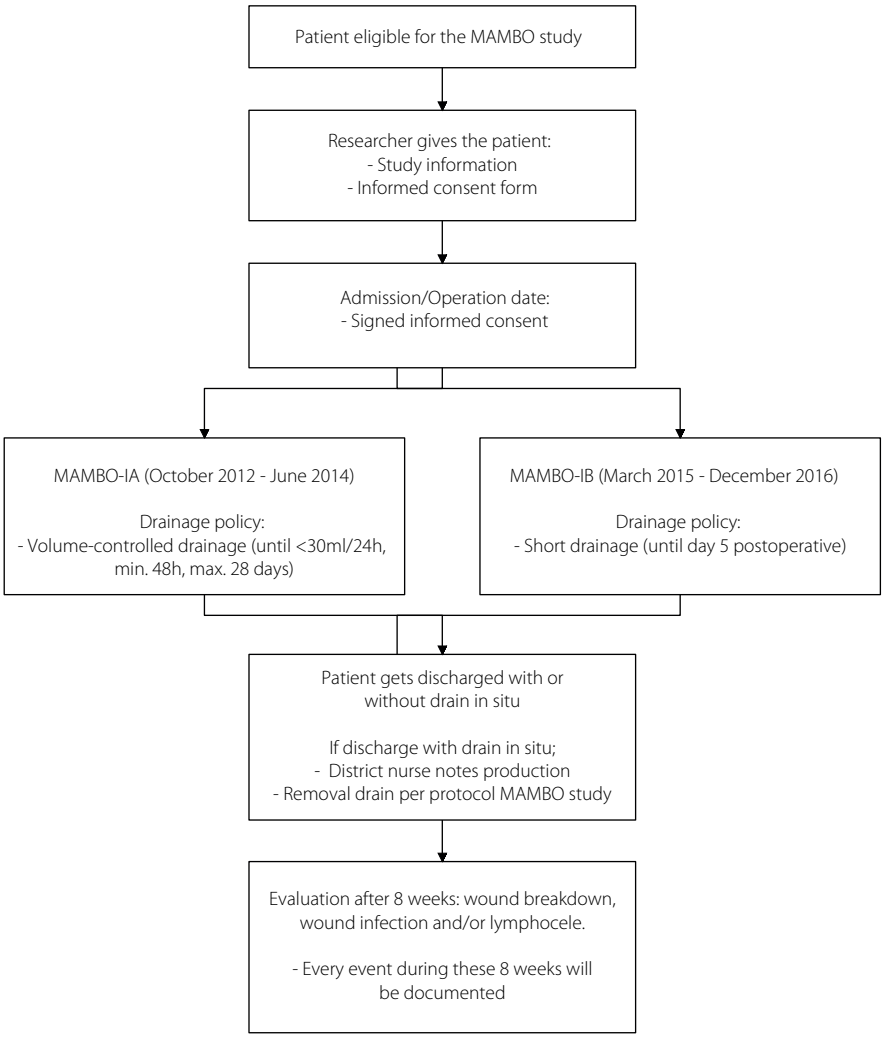

Figure 1 Summary of the MAMBO study protocol

Abbreviations: IFL = inguinofemoral lymphadenectomy, ml = milliliters, ml/24h = milliliters per 24 hours

RESULTS

We included 141 patients (251 groins) ; 77 patients (139 groins) for volume-controlled drainage (MAMBO-IA) and 64 patients (112 groins) for short drainage (MAMBO-IB). Table 1 shows that the two groups were similar in terms of patient and treatment characteristics.

Volume-controlled drainage

In Figure 2 is shown that the median duration of drainage of the groin was 13 days (range 2-40 days) and the median production on the last day of drainage was 25 ml (range 0-720 ml). The duration of hospital stay was median five days (range 1-22 days) and 57/77 (74%) patients went home with at least one drain in situ.

In 79/139 (57%) groins in the volume-controlled drainage group, the drain was removed in deviation of the criteria prescribed in the protocol. In 34/79 (43%) groins, drainage was a shorter period than prescribed (removal before the production of <30 ml/day) and in 39/79 (49%) groins drainage was performed for a longer period (removal after the production was < 30 ml/day). In 6/79 (8%) groins, it was unclear when the drain was removed.

Reasons for drainage for a shorter period than prescribed were: in 12 groins wound complications (infection or dehiscence), in five groins drain problems (pain, clotted drain) and in 13 groins drain fell out spontaneously. In groins that were drained for a longer period than prescribed; this was mainly (36/39, 92%)) due to logistic difficulties (homecare nurse did not remove the drain, patients waited until their appointment at the outpatient clinic and/or doctors/nurses were not sufficiently familiar with the research protocol).

Short drainage

For groins in the short drainage group, the median duration of drainage was five days (range 3-24 days) with a median production of 60 ml (range 0-380 ml) on the last day of drainage, see Figure 3. The median duration of hospital stay was six days (range 1-26 days) and 10/64 (16%) patients were discharged with at least one drain in situ.

The drain was removed in deviation of the protocol in 13/112 (12%) of the groins. Of these groins, 8/13 (62%) were drained longer and 5/13 (38%) were drained shorter than five days. Reasons for longer drainage were in two groins high drain production on day five, in the other groins the reasons were unknown. Five groins were drained shorter than five days; in two groins the drain fell out spontaneously and in one groin the drain was removed due to pain. For the other two, the reason for early removal was unknown.

Table 1 Patient-, tumor-, and operation characteristics of the study population

	Volume-controlled drainage N = 77 patients, N = 139 groins		Short drainage N = 64 patients, N = 112 groins	
	Median (range)	N (%)	Median (range)	N (%)
Patient characteristics				
Age (years)	68 (35-88)		70 (43-89)	
BMI (kg/m²)	27.6 (19.4 – 44.1)		27.6 (18.5-43.0)	
Diabetes mellitus				
- Yes		13 (17)		8 (13)
- No or unknown		64 (83)		56 (87)
Smoking				
- Active		12 (16)		13 (20)
- Not		62 (81)		43 (67)
- unknown		3 (3)		8 (13)
Tumor characteristics				
Diameter tumor (in mm)	30.0 (7-100)		35.0 (2.4-100.0)	
Location tumor				
- Central (≤1 cm from midline)		63 (82)		43 (67)
- Lateral (>1cm from midline)		13 (17)		18 (28)
- Unknown		1 (1)		3 (5)
Invasion depth (in mm)	5.0 (1.5-33)		6.0 (1.0-25.0)	
Focality				
- Unifocal		63 (82)		46 (72)
- Multifocal		13 (17)		17 (27)
- Unknown		1 (1)		1 (2)
LVSI				
- Present		17 (22)		16 (25)
- Absent		57 (74)		44 (69)
- Unknown		3 (2)		4 (6)

Lymph nodes			
Number of nodes removed per groin	8.0 (1-21)	7.0 (0-20)	
Groins with positive nodes		33(25)	35 (32)
Operation			
Type of IFL			
- Bilateral		62 (81)	48 (75)
- Unilateral		15 (19)	16 (25)
Order of procedures			
- Excision vulva and IFL in same procedure		60 (78)	38 (59)
- IFL only		16 (21)	26 (39)
- Unknown		1 (1)	2 (2)
Closure technique per groin			
- Staples		60 (43)	30 (27)
- Stitches		1 (1)	27 (24)
- Intracutanous		42 (30)	46 (41)
- Combination of above or unknown		36 (26)	9 (8)
Variation of the surgical technique per groin			
- Ligation saphenous vein		20 (14)	4 (4)
- Sartorius transposition		2 (1)	0 (0)
Prophylactic antibiotics			
- Yes		74 (96)	63 (98)
- No		1 (1)	0 (0)
- unknown		2 (3)	1 (2)

Footnote: N=total number of patients or groins, BMI=body mass index, IFL=inguinofemoral lymphadenectomy, mm=millimetres, cm=centimetres, LVSI=lymph-vascular space invasion

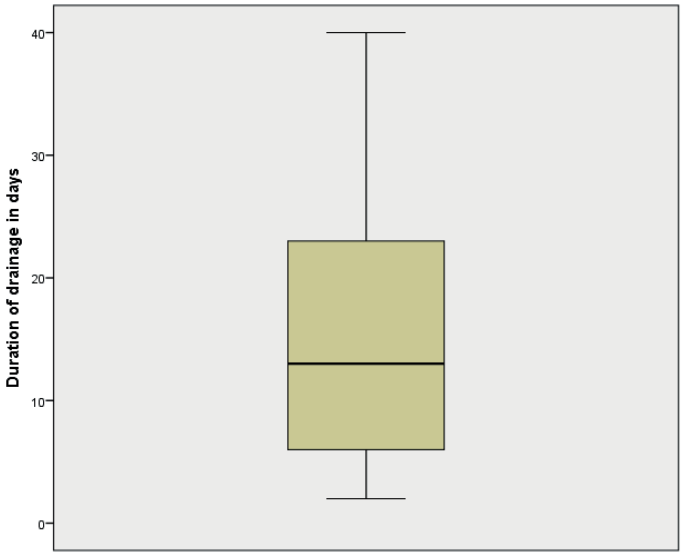

Figure 2 Duration of drainage in days after volume-controlled drainage

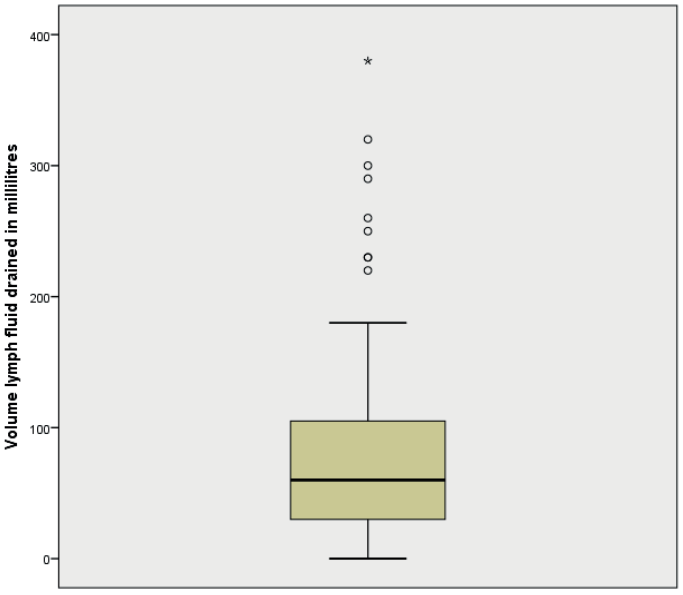

Figure 3 Volume of drained lymph fluid in milliliters on the last day in situ after short drainage

Intention-to-treat analysis

Short-term complications per patient

The estimated incidences of short-term complications after volume-controlled versus short drainage are shown in Table 2. The estimated incidence of a lymphocele was 16% after volume-controlled drainage versus 60% after short drainage (RD 45% (95% CI 30, 59), p<0.001). The estimated incidence of a wound infection was 52% in both drainage groups and primary wound dehiscence of 8% after volume-controlled drainage versus 11% after short drainage (RD 3% (95%CI -7, 13) p=0.534). The adjusted incidence of one or more complications (wound infection, primary wound dehiscence or lymphocele) was 67% after volume-controlled drainage versus 91% after short drainage (RD 24% (95% CI 7, 41), p=0.006).

After volume-controlled drainage, 36/77 (47%) patients with a complication needed treatment such as antibiotics, incision and drainage, wound exposure, necrotectomy, or a combination of these treatments. After short drainage 42/64 (66%) of the patients needed any treatment for a complication. In total 21/77 patients (27%) after volume-controlled drainage needed to be readmitted because of a complication versus 21/64 (33%) after short drainage.

Secondary wound healing, as a result of one of the previous described complications or the treatment given for a complication, was present in 25/77 (32%) patients after volume-controlled drainage versus in 33/64 (52%) after short drainage.

Short-term complications per groin

Table 2 shows the estimated incidences of short-term complications per groin after volume-controlled versus short drainage; it shows an estimated incidence of lymphoceles in 10% of the groins after volume-controlled drainage compared to 52% after short drainage (RD 42% (95% CI 31, 52), p < 0.001). The estimated incidence of a wound infection was 40% after volume controlled drainage versus 43% after short drainage (RD 3% (95% CI -9, 15, p = 0.650), the incidence of primary wound dehiscence was estimated in 5% versus 7% of the groins respectively (RD 2% (95% CI -4, 8), p = 0.565). One or more complications (wound infection, primary wound breakdown or lymphocele) per groin was estimated, after adjustment, in 46% of the groins after volume-controlled drainage versus 75% after short drainage (RD 29% (95% CI 8, 49), p=0.006).

Secondary wound healing, as a result of one of the previous described complications or the treatment given for a complication, was present and in 42/139 (30%) after volume-controlled drainage versus 52/112 (46%) after short drainage. After 8 weeks of follow up, 110/139 (79%) of the groin wounds were healed in the volume-controlled drainage group versus 68/112 (61%) in the short drainage group.

Table 2 Estimated incidence of short-term complications after volume-controlled versus short drainage; intention-to-treat analysis

	Volume-controlled drainage % (95% CI)	Short drainage % (95% CI)	Risk difference RD (95% CI), p-value
Per patient	N = 77	N = 64	
Wound infection	52% (41, 63)	52% (39, 64)	0% (-17, 16) p = 0.959
Primary wound dehiscence	8% (2, 14)	11% (3, 19)	3% (-7, 13) p = 0.534
Lymphocele	16% (8, 25)	60% (48, 72)	45% (30, 59) p < 0.001
≥1 complication*	**67% (44, 84)**	**91% (71, 98)**	**24% (7, 41) p = 0.006**
Per groin	N=139	N=112	
Wound infection	40% (32, 48)	43% (34, 52)	3% (-9, 15) p = 0.650
Primary wound dehiscence	5% (1, 9)	7% (2, 12)	2% (-4, 8) p = 0.565
Lymphocele	10% (5, 15)	52% (43, 61)	42% (31, 52) p < 0.001
≥1 complication*	**46% (9, 88)**	**75% (24, 97)**	**29% (8, 49) p = 0.006**

Table 3 Estimated incidence of short-term complications after volume-controlled versus short drainage; per-protocol-analysis

	Volume-controlled drainage % (95% CI)	Short drainage % (95% CI)	Risk difference RD (95% CI), p-value
Per groin	N=52	N=99	
Wound infection	32% (19, 45)	45% (36, 55)	13% (-3, 30) p = 0.104
Primary wound dehiscence	2% (-2, 6)	7% (2, 12)	5% (-1, 11) p = 0.119
Lymphocele	10% (2, 18)	54% (44, 63)	44% (31, 56) p < 0.001
≥1 complication*	**30% (2, 88)**	**71% (11, 98)**	**41% (16-64) p < 0.001**

Per-protocol analysis

Short-term complications per groin

For groins treated to the study protocol, the estimated incidence of complications is shown in Table 3. The estimated incidence of a lymphocele was 10% after volume-controlled drainage versus 54% after short drainage (RD 44% (95% CI 31, 56), p<0.001). In addition, the incidence of wound infection (32% versus 45%) and primary wound dehiscence (2% versus 7%) did not significantly differ between the two groups. The adjusted incidence of one or more complications was 30% in the volume-controlled drained groins versus 71% in the short drained groins (RD 41% (95% CI 16, 64), p<0.001).

Drainage longer or shorter than the study protocol

In Figure 4 is shown that the incidence of complications differs between groins drained for a longer period or for a shorter period than prescribed by the study protocol.

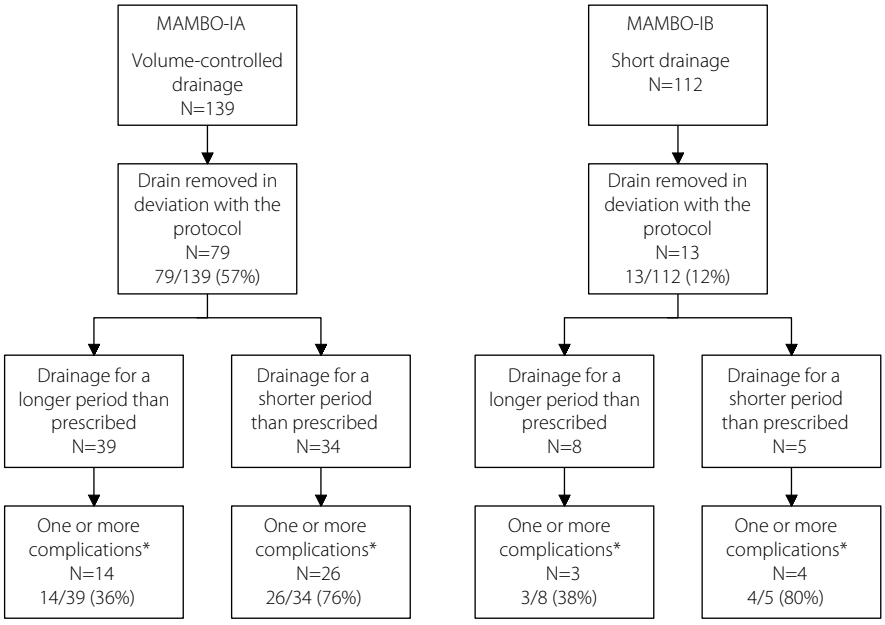

Figure 4 Incidence of complications per groin displayed by drainage for a longer or shorter period than described by the volume-controlled or short drainage study protocol

Footnote: * including wound infection, wound breakdown, lymphocele

In the volume-controlled drainage study group, 39/79 (49%) of the groins were drained for a longer period than the protocol described and 34/79 (43%) groins for a shorter period. In the longer drained groins, one or more complications were present in 14/39 (36%) of the groins; in the shorter drained groins 26/34 (76%). However, in these shorter drained groins reasons for early removal was a complication in 12 groins.
In the short drainage study group 8/13 (62%) groins were drained longer than 5 days and 5/13 (38%) were drained shorter. One or more complications were present in 3/8 (38%) of the longer drained groins versus 4/5 (80%) of the shorter drained groins. In none of the groins which were drained shorter than prescribed a complication was the reason for removal.

DISCUSSION

Our study is the first prospective study concerning drain management after IFL in vulvar cancer patients. We show that volume-controlled drainage results in a significant reduction of complications compared to short drainage; this difference was mainly related to less lymphoceles. Moreover, the incidence of postoperative wound infections and wound breakdown did not significantly differ between the two groups. Moreover, this study showed that both drainage protocols are feasible; however, the volume-controlled drainage protocol is more difficult to follow. As this study shows the advantages of volume-controlled drainage in terms of a significant reduction of complications, we propose introducing volume-controlled drainage as a standard protocol after IFL.
This study showed that in the volume-controlled treatment group, only 37% of the groins were drained according to the study protocol. Therefore, we performed both an intention-to-treat and per-protocol analyses. However, the intention-to-treat analysis reflects clinical practice.
As shown in Figure 4, the incidence of complications differs between groins drained according protocol or for a shorter or for a longer period than prescribed. In the volume-controlled drainage study group, the lowest complication rate (one or more complications) was after drainage according to the protocol (estimated incidence 30%) or for a longer period (14/39, 36%); the highest complication rate was in groins drained for a shorter period than prescribed (26/34, 76%). However, this shorter period of drainage does not fully explain the higher complication rate, as one third of the drains which were removed earlier than the protocol prescribed were removed because of a complication. In conclusion, this study shows that drainage for a longer period, does not seem to harm the patient in terms of complications. However, the longer the duration of drainage, the longer the patient experiences discomfort.

This study was not designed to define an optimal cut-off level for drain production and removal of the drain in which the complication rate is lowest. However, the median production of the drain on the last day in situ after short drainage was 60 ml (see Figure 3). As short drainage was associated with complications in 91% of the patients, we expect the optimal cut-off level for removal of the drain to be at least below 60 ml. Not much literature has been published on drainage protocols after IFL in vulvar cancer. In addition to the indication for vulvar cancer patients, IFL is also commonly performed when treating melanoma patients. In these patients, early wound complication rates are reported in 50% - 72% of cases [15, 16]. As shown in our study and reported in the literature, the complication rate in vulvar cancer patients is much higher than in melanoma patients. This difference can be partly explained by patient characteristics, as patients in our study had a higher median age (10 years) than melanoma patients, and several have reported older age as a statistically significant risk factor for a complication [15, 16]. Additionally, it is likely that the higher the patients age, the greater the presence of co-morbidity. As co-morbidity, such as diabetes, affects the complication rate, this could also explain the difference in complication rates between vulva cancer and melanoma patients.

Besides, lymphadenectomy is also performed in the axilla. In patients treated for breast cancer, the postoperative drain management after axillary lymphadenectomy has been studied in more detail. It covers different aspects of drain management after axillary lymphadenectomy; the need for suction drainage, the amount of negative pressure and the duration of drainage. These studies concluded that suction drainage is mandatory in decreasing lymphocele formation, but that the different amounts of pressure applied is of less value [17-20]. A trend towards decreased lymphocele formation and a decrease in wound healing problems after closed suction drainage was reported [21-24]. Furthermore, several studies and a meta-analysis compared early with late drain removal and concluded that early drain removal was safe, but the incidence of lymphoceles tends to be higher and reported no difference in the incidence of wound infections [25-31].

There are differences in the treatment of axillary lymphoceles and groin lymphoceles. In axillary lymphoceles (repeated) percutaneous drainage is performed. As the anatomy of the groin and axilla differs, the treatment of groin lymphoceles is more complex; this is possible due to more pockets of lymph fluid in the groin. Therefore, it is important to prevent formation of a groin lymphocele. As previously described, both literature on breast cancer and our study showed less lymphocele formation using volume-controlled drainage.

The past decades, new surgical techniques for IFL were implemented. This resulted in a decrease of the incidence of complications. However, even after using the volume-controlled drainage, the incidence of complications still remains considerable. Future research should focus on other factors that may play a role in complications

after IFL. Besides postoperative care, modifying the surgical technique might be the key factor to decrease the complication rate.

A novel and promising surgical technique is video endoscopic inguinal lymphadenectomy (VEIL). A study in penile cancer patients compared the open procedure versus VEIL in 30 groins (21 patients) and reported complications (skin related events, lymphatic complications, hematoma) in 20% of the groins that underwent VEIL versus 70% in open procedure groins (p=0.015). The average number of hospitalization days was reduced from 6.4 days to 24 h after VEIL (p<0.001) [32]. In 21 patients with vulvar cancer undergoing VEIL a complication rate (wound necrosis, lymphocele) of only 4.8% is reported [33]. These results show a much lower complication rate compared to our study population. VEIL appears to be a promising technique to decrease the short-term morbidity, although there is a need for more data regarding oncological safety of this method.

In conclusion, this nationwide prospective study showed that mainly due to the reduction in lymphoceles, volume-controlled drainage of the groin after IFL is feasible and is associated with a significant reduction in the incidence of one or more short-term complications compared to short drainage. We therefore propose that volume-controlled drainage after IFL becomes the standard protocol for vulvar cancer patients. However; the incidence of complications still remains considerable. To further elucidate the emerging and remaining questions future research might focus on surgical techniques and/or other postoperative care protocols to define the optimal care for patients with an indication for IFL in order to further reduce the short-term complication rate.

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Reduced morbidity by using LigaSure compared to conventional inguinofemoral lymphadenectomy in vulvar cancer patients; a randomized controlled trial

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Submitted

ABSTRACT

Objective IFL is part of the surgical treatment of different malignancies of the genital tract and/or the lower limb including vulvar carcinoma, penile carcinoma and melanoma. IFL is associated with morbidity in up to 85% of the patients. The aims of this MAMBO-IC study (Morbidity And Measurement of the Body) are to study the feasibility of using LigaSure for inguinofemoral lymphadenectomy (IFL) and to assess the differences in the incidence of short-term complications using LigaSure versus conventional IFL randomized within each individual patient.

Methods In this multicenter randomized controlled trial (RCT), women diagnosed with squamous cell carcinoma of the vulva with an indication for bilateral IFL were included. It was randomly assigned for which groin the LigaSure was used; the other groin was treated with conventional IFL (sharp/diathermia). We estimated the incidence of ≥ 1 complication(s) per groin using logistic regression and compared this between the two surgical methods, adjusting for possible confounders.

Results We included 40 groins of 20 patients. The estimated incidence of ≥1 complication(s) was 29% after LigaSure versus 70% after conventional IFL (risk difference 41% (95% CI 19-62), $p < 0.001$). Patients' reported restriction of daily living activities and maximum pain score were equal for both treatment methods. There were no differences in the surgeon reported workload scores.

Conclusions This RCT shows that LigaSure for IFL is feasible and associated with significantly less short-term surgical complications compared to conventional IFL. Further studies with a larger sample size are needed to validate our findings.

INTRODUCTION

Inguinofemoral lymphadenectomy (IFL) is part of the surgical treatment of malignancies of the genital tract and/or the lower limb such as vulvar carcinoma, penile carcinoma and melanoma. This procedure is associated with surgery related morbidity in up to 85% of the patients. This morbidity concerns short-term (wound infection, formation of lymphoceles, wound breakdown) and long-term morbidity (lymphedema, cellulitis, erysipelas) [1-4]. Advanced age and comorbidity including diabetes are risk factors for postoperative complications after IFL [3, 5, 6].

We have demonstrated in two consecutive national prospective studies 'Morbidity And Measurement of the Body' (MAMBO-IA and IB) that volume-controlled drainage of the groin resulted in a reduced short-term complication rate when compared to short drainage. Nevertheless, complications are still present in 67% of the patients after volume-controlled drainage, and 53% of these patients needed to be readmitted to the hospital [7]. As a consequence, these complications lead to a significant increase in health care costs, which supports the urgent need for additional effort to reduce surgical morbidity.

Adaptations in the surgical approach may play a key role in reducing morbidity associated with IFL. Several modifications in the surgical approach have been explored, such as separate incisions, unilateral IFL, sparing of saphenous vein, preservation of the fascia lata and continuous skin closure, but only a limited reduction of morbidity was achieved [8, 9]. Last decade, new surgical devices have been developed including: energy-based ultrasonic, bipolar vessel or electrothermal vessel sealing. These devices can seal blood and lymph vessels, and may subsequently reduce postoperative leakage of lymph fluid which may reduce surgical morbidity. Although these new surgical devices are increasingly used in clinical practice in addition to conservative surgery (sharp knife dissection, mono- or bipolar electrocautery), comparative studies are limited.

In one randomized controlled trial (RCT) published as a conference abstract, harmonic scalpel was compared with clips, electrosurgery or ligatures in IFL. In this study less postoperative complications (lymphocele, wound breakdown or infectious complications requiring surgery) were reported in the harmonic scalpel group (38%) compared to the control group (61%), p=0.059. Unfortunately, this study was interrupted after 61 instead of the sample size of 125 patients due to slow accrual and therefore underpowered [10]. RCTs comparing LigaSure versus conventional axillary dissection in breast cancer patients reported significant less intra-operative blood loss, reduction in the amount of drained lymph fluid, less days of suction drainage and shorter hospitalization in patients treated using LigaSure. No differences were reported in the rate of hematomas, reoperations or infections [11-13].

In the current RCT (MAMBO-IC) we aim to study the feasibility of LigaSure for IFL and to assess the incidence of short-term complications, using LigaSure versus the conventional performance of IFL randomized within each individual patient. Moreover we will evaluate patients' and surgeons' experience.

METHODS

Patients

We conducted this RCT, the MAMBO-IC study (MAMBO: Morbidity And Measurement of the BOdy), in two gynecologic oncology centers: the Radboud university medical center and the University Medical Center Groningen. All patients aged ≥ 18 years with vulvar squamous cell carcinoma (SCC) with an indication for bilateral IFL were eligible for inclusion. Patients were excluded if they received radiotherapy to the vulva, groins and/or pelvis previously, pelvic lymphadenectomy, or if there was an indication for IFL with the 'en bloc' approach or other histology than SCC. Patients were informed about this study and approached to participate during a regular visit at the outpatient clinic. Written informed consent was obtained from all included patients before enrollment. The study was conducted according to the principles of the Declaration of Helsinki (2008) and to the Medical Research Involving Human Subjects Act (Dutch: WMO). The study protocol was medical-ethically approved to be conducted by the Medical Ethical committee of Arnhem-Nijmegen (NL62326.091.17), and registered in the ISRCTN registry (ISRCTN15057626).

Randomization process

Patients were randomized to the intervention (LigaSure™ Small Jaw Open Sealer/ Divider LF1212A (Medtronic)) for either the left or right groin with a 1:1 allocation ratio. We used a variable block randomization method, with a block division of 2, 4 using Castor EDC. The outcome of randomization was blinded for the patients, doctors, nursing staff and caregivers, except for the performing surgeons.

Surgical procedure

All patients underwent a bilateral IFL. The LigaSure devices were partly provided by the manufacturer. It was randomly assigned for which groin the LigaSure was used to perform this surgical procedure and for the other groin, the conventional method (scalpel and/or electrocautery) was used. Preoperative antibiotic prophylaxis consisted of 2000mg Cefazoline and 500 mg Metronidazole intravenously. The IFL was a standard procedure as described previously [14]. A high vacuum Redon drain (775 mmHg, 0.9 bar negative pressure) was placed in the groin just before closure. None of the surgeons was trained specifically for using LigaSure. IFLs were performed either

subsequently by the same surgeon or, to reduce operating time, simultaneously by two different surgeons.

Postoperative care

The groin drain was removed when the production of the drain is ‹30ml/day with a minimum of three days, according to the MAMBO-IA protocol [7]. After each surgical procedure, the surgeon was requested to complete the online questionnaire regarding their experience regarding the surgical procedure containing SURG-TLX [15]. In the SURG-TLX, surgeons rate six dimensions of workload: mental-, physical- and temporal demands, task complexity, situational stress, and distractions, on a 20-point Likert scale, anchored between low and high.

Follow-up

Follow-up for the study was completed eight weeks after surgery. Patients were routinely seen at two and eight weeks after surgery by a gynecologic oncologist, both groins were examined and any complication was reported. All included patients were approached at eight weeks postoperatively by the investigator to complete a telephone questionnaire regarding pain and restriction of daily activities. The maximum postoperative pain and the restriction of daily activities was scored on a visual analogue scale (VAS) between zero (low) and ten (high).

Outcomes

The primary objective was to study the feasibility of LigaSure and to determine the incidence of any short term complication i.e.: wound breakdown and/or wound infection and/or lymphocele, within eight weeks after IFL after using LigaSure or the conventional method. Wound breakdown was defined as every spontaneous disrupted groin wound › 2cm, wound infection as purulent exudates and/or positive culture and/or erythema and lymphocele as the collection of lymph fluid › 5cm.
The secondary objectives were to determine the differences between the two surgical methods in duration of drainage and volume drained, operating time, to evaluate patients' experience regarding postoperative pain and restriction of daily activities, and to evaluate the surgeon's experience of both surgical procedures using a questionnaire.

Statistical analysis

The incidences of wound infection, primary wound dehiscence and lymphocele are displayed by frequencies and compared with the McNemar test. To estimate the incidence of ≥1 complication per groin, we used a generalized linear model with a logit link and a binomial distribution, and a random effect for patient. In order to quantify the differences between conservative IFL and Ligasure, risk differences were estimated using this model, adjusted for the number of lymph nodes removed per groin. Continuous variables are

summarized using the median and range and compared between the two treatments using the Wilcoxon Signed-rank test. Discrete variables were described by frequencies. Two-sided p-values <0.05 were regarded as statistically significant. Analyses were performed using SPSS version 22.0 [16].

RESULTS

Twenty patients with 40 groins were included and randomized in this MAMBO-IC study; ten patients were allocated to IFL with LigaSure in the left groin and ten patients to IFL with LigaSure in the right groin. The IFLs were performed between October 2017 and October 2018 in either the Radboudumc (N=9) or the University Medical Center Groningen (N=11). Patient- and tumor characteristics are shown in Table 1, and did not notably differ between the two treatment centers. The indication for bilateral IFL was a tumor with a diameter of more than 4 cm in eight (40%) patients, pathologically proven lymph node metastases in five patients (25%), a positive SLN in three patients (15%) or local recurrent disease (without earlier IFL in primary treatment) in four patients (20%). In 17 patients, the IFL was performed concomitantly with vulvar surgery, and in three patients there was a positive SLN in the treatment of the current primary carcinoma and IFL was performed in a second procedure ranging between 34 and 60 days after the SLN procedure. The IFLs were performed by 11 different surgeons without differences in the applied standardized surgical technique, the number of dissected groins per surgeon ranged between one and 11.

Surgical outcomes

The median duration of the IFL was 56 minutes (range 27-105) for groins dissected with LigaSure and 57 minutes (range 36-90) for groins dissected conservatively, p=0.570. The median number of lymph nodes removed per groin was 9.5 (range 2-18) in groins treated with LigaSure versus median 10 (range 5-14) in groins treated by the conservative IFL, p=0.692. Operative characteristics are summarized in Table 2.

Postoperative outcomes

The duration of drainage did not differ between LigaSure compared to conservative IFL, median 16.5 days (range 5-54) versus median 19.5 days (range 4-34) respectively, p=0.727. The median volume drained per day for each treatment group is displayed in Figure 1. The total volume drained during the period of drainage was not significantly different between groins treated by LigaSure or conservative IFL, median 1037 ml (range 200-5030) and median 1533 ml (range 325-6020) respectively, p=0.156. Analyzing only groins drained according protocol (N=21), the total drain production was significantly lower in the groins treated by LigaSure compared to conservative IFL,

Table 1 Patient- and tumor characteristics of the study population

	Median (range)	N (%)
Patient characteristics		
Age (years)	75 (53-88)	
Body mass index (kg/m²)	29.3 (22.5-42.0)	
Diabetes Mellitus		
Yes		3 (15)
No		17 (85)
Smoking		
Yes		3 (15)
No		13 (65)
unknown		4 (20)
Tumor characteristics		
Diameter (mm)	37.5 (9-80)	
Location tumor		
Central		19 (90)
Lateral		2 (10)
Depth of invasion (mm)	(1.7-21.0)	
Focality		
Unifocal		20 (100)
Multifocal		0 (0)
Lymphovascular space involvement		6 (30)
Yes		13 (65)
No		1 (5)
unknown		
Pathological tumor free margins		
Yes		19 (95)
No		1 (5)
Presence of precursor lesion		
None		5 (25)
HSIL		0 (0)
LS		3 (15)
dVIN		5 (25)
dVIN and LS		7 (35)

median 1180 ml (range 750-2760) and median 1885 ml (range 1430-3340) respectively, p=0.043 without a difference in the duration of drainage between the two treatment groups, p=0.180.

Table 2 Operative characteristics of the included groins

	LigaSure (N=20)		Conservative(N=20)	
	Median (range)	N (%)	Median (range)	N (%)
Duration of procedure (minutes)	56 (27-105)		57 (36-90)	
Method of closure				
Intracutanous		5 (25)		5 (25)
Stitches and intracutanous		4 (20)		4 (20)
Staples		11 (55)		11 (55)
Previous SLN procedure				
Yes		7 (35)		4 (20)
No		13 (65)		16 (80)
Number of lymph nodes removed per groin	9.5 (2-18)		10.0 (5-14)	
Number of groins with lymph nodes metastases		9 (45)		7 (35)

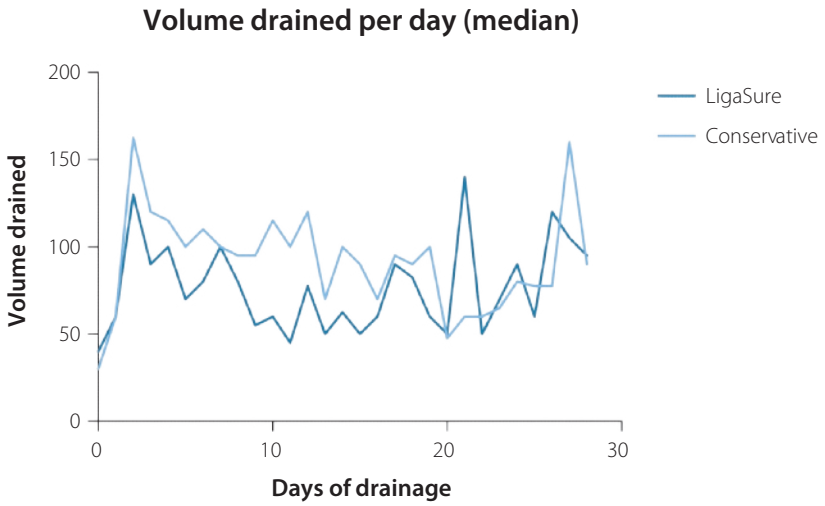

Figure 1 Volume drained per day by surgical method used for IFL

Table 3 (Estimated) incidence of short-term complications per groin

Incidence of short-term complications per groin			
	Conservative N=20	LigaSure N=20	p-value
Wound infection	50%	30%	p = 0.125
Primary wound dehiscence	5%	5%	p = 1.0
Lymphocele	25%	20%	p = 1.0
Estimated incidence of short-term complications per groin			
	Conservative	LigaSure	Risk Difference (95% CI)
≥1 complication*	70% (95% CI 47-85)	29% (95% CI 13-52)	41% (95% CI 19-62), p < 0.001

*adjusted for number of lymph nodes removed

Short-term complications

The incidence of a wound infection, primary wound dehiscence and lymphocele per groin did not differ between the two surgical treatment methods. The estimated incidence of ≥1 complication per groin was 29% (95% CI 13-52) after LigaSure compared to 70% (95% CI 47-85) after conservative IFL (RD 41% (95% CI 19-62)), p < 0.001. See table 3 for an overview of the outcomes.

One or more complications of the groin occurred in 70% (14/20) of the patients and are equally distributed between the two treatment centers. As shown in Figure 2, in six patients a bilateral complication was present, and in eight patients an unilateral complication. In all patients with a unilateral complication, the groin in which the complication occurred was treated by conservative IFL.

In 6/20 (30%) groins, treatment was necessary following a short-term complication after LigaSure. In groins treated with conservative IFL, treatment was given in 11/20 (55%) of the groins. Eight (8/20; 40%) patients were readmitted because of a complication of the groin with a median duration of 7.5 days (range 3-26). Four of these patients had a bilateral short-term complication of the groin, and four a unilateral complication. Secondary wound healing was reported in four groins of two patients. Adjuvant radiotherapy to the groins was given to 11/20 (55%) patients and postponed due to groin complications in 2/11 (18%) patients.

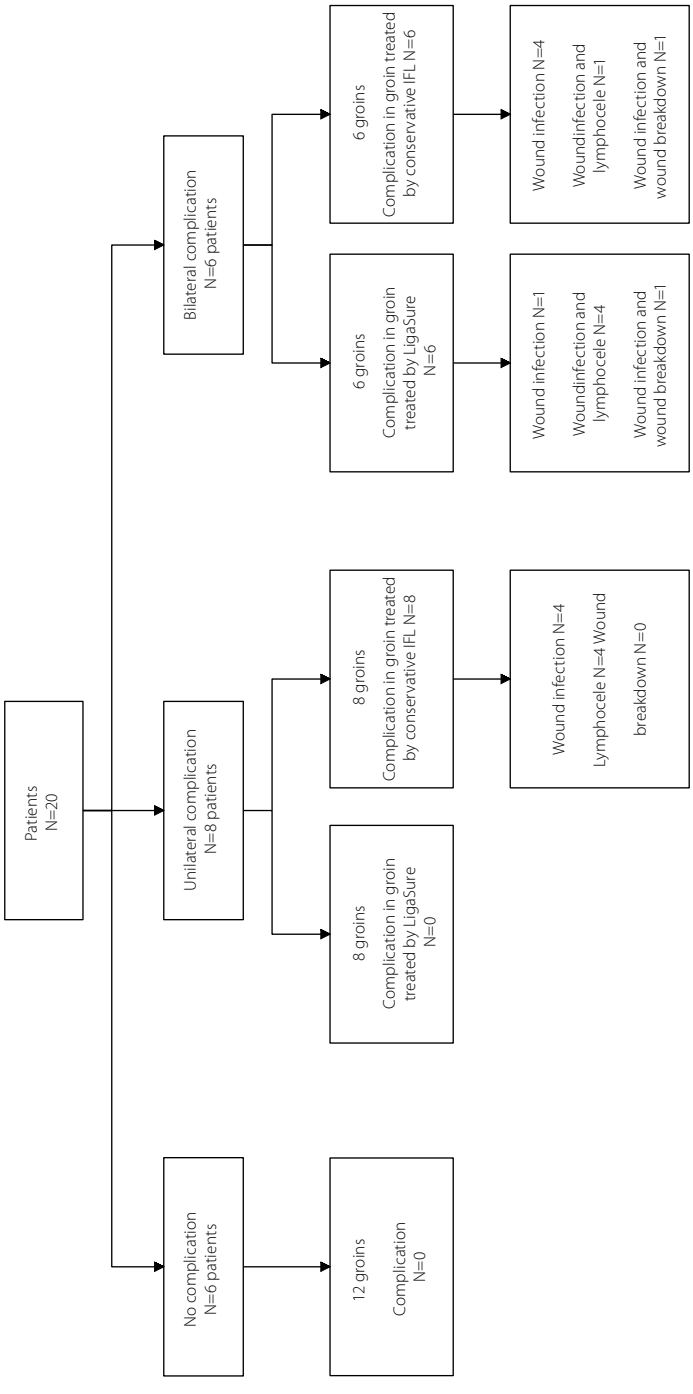

Figure 2 Short-term complications of the groin stratified by surgical method used for IFL

Surgeons' experience

Figure 3 shows the workload scores for each dimension for both surgical methods. There were no notable differences in the surgeons' reported workload scores. Of the surgeons performing IFL using LigaSure, 75% had used this device previously. All surgeons who performed the conservative IFL would recommend this method to their colleagues, versus 15/20 (75%) surgeons who used LigaSure. Reasons for not recommending LigaSure to their colleagues were: conservative method is more easy, LigaSure is less precise compared to conventional IFL, prefers to use a combination of both LigaSure and the conservative method.

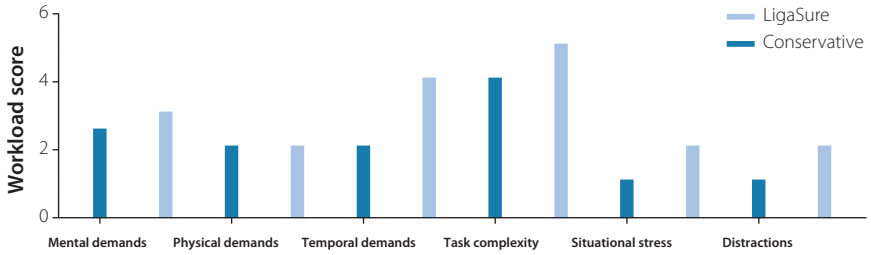

Figure 3 Surgeons' workload score (median) by surgical method used for IFL

Patients' experience

Restriction of daily activities and maximum pain score were scored equally by the patients for both treatment groups. After LigaSure, the maximum pain score was median 3 (range 0-8) and after conventional IFL median 3 (range 0-9), p=0.844. The restrictions of daily activities was scored median 0 (range 0-8) after LigaSure versus median 0 (range 0-8) after conservative IFL, p=0.655.

DISCUSSION

This multicenter RCT is the first study comparing LigaSure and conventional IFL in vulvar SCC patients. The use of LigaSure for IFL is feasible and significantly reduce the estimated incidence of ≥1 complication(s) per groin compared to conventional IFL. However, the incidence of a wound infection, primary wound dehiscence and lymphocele per groin did not differ between the two surgical treatment methods, probably due to lack of power. The duration of surgery, number of removed lymph nodes, duration of drainage and volume drained did not notably differ between the two surgical methods. In addition, the patients' and surgeons' experience did not differ between both treatment methods.

Although RCTs comparing LigaSure versus conventional axillary dissection in breast cancer patients reported reduced volume drained, shorter period of drainage and reduction of duration of postoperative hospital stay after LigaSure [11-13], this study did not confirm these results in vulvar SCC patients. However, in groins drained according protocol, the total drain production was significantly lower in the groins treated with LigaSure compared to groins treated with conservative IFL, without a difference in the duration of drainage. Due to our study design, with both treatments randomized within a patient, we were not able to assess the difference in days of hospital stay or readmission for the two surgical methods.

Using LigaSure, the simultaneous sealing and cutting of the vessels and tissue without the need of changing instruments might reduce the operating time. In breast cancer patients, the use of LigaSure for axillary lymphadenectomy significantly reduced the operating time with 15 minutes in a study including 100 women randomized for either LigaSure or conventional axillary lymphadenectomy [11].

In our study, there was no difference in operating time comparing Ligasure versus conventional IFL. In contrast, Pellegrino *et al.* [17] reported, in a study comparing the harmonic scalpel to conventional electrosurgery in 42 patients with vulvar cancer, a significant reduction of 25 minutes in operating time in favor of the harmonic scalpel. Operating time included radical local excision of the tumor combined with uni- or bilateral IFL. The reduction of operating time might be partially due to the use of the harmonic scalpel for the wide local excision of the vulvar tumor.

Although the introduction of the SLN procedure has become a big step forward in terms of reduced morbidity, IFL will always keep a place in the treatment of vulvar SCC patients, eg. in patients with a multifocal tumor and/or a diameter more than four centimeters. Therefore, attempts should be made to further reduce postoperative morbidity. In previous years, minimally invasive techniques are developed to reduce postoperative morbidity of IFL and promising results are published. A systematic review including nine retrospective studies and 249 video endoscopic inguinofemoral lymphadenectomy (VEIL) procedures reported a complication in 6% of the groins,

including a lymphocele in 3.6%, wound infection in 1.2% and lymphedema in 0.4% of the groins [18]. Recently, an RCT, randomizing for either VEIL with the limb subcutaneous approach (N=8) or with the hypogastric approach (N=17), compared the postoperative morbidity to a historical cohort of 21 patients undergoing open IFL showed significantly less complications in the groin were reported after VEIL (infection 8% vs 19%, lymphocele 8% versus 10%, wound dehiscence or skin necrosis 0% versus 14%) [19]. In contrast, one study including 12 patients and 22 groins reported the rate of ≥1 complication after robot-assisted VEIL (VEIL-R): 59% per groin and 75% per patient [20]. In spite of the small number of patients, these complication rates are even higher compared to the rate of ≥1 complication in previously reported studies after open IFL [7, 21]. In addition, none of the mentioned studies included Caucasian women, and neither reported data concerning the oncologic safety of this procedure. A large prospective trial with adequate follow- up of at least 2 years is needed to determine the oncologic safety of VEIL and to determine the postoperative morbidity in women with vulvar SCC. In addition, VEIL is preferably compared to open IFL using either a energy-based ultrasonic, bipolar vessel or electrothermal vessel sealing device, as the device used might be key to lowering postoperative morbidity.

Based on the results of this study, the use of LigaSure is feasible to perform IFL and the surgeons' workload was equal. However, treatment with LigaSure leads to additional costs (about €250/$285 per disposable device) compared to the less costly diathermia and/or scalpel. These additional costs are counterbalanced by reduced costs of postoperative care, such as treatment of complications and readmissions, as result of reduced surgical morbidity after IFL using LigaSure. Future research including a cost-effectiveness analysis may give the answer if LigaSure is cost-effective in the treatment of vulvar SCC patients.

Besides the obvious limitation of a small number of included patients, the surgical procedures in our study were performed by different surgeons. However, this reflects daily clinical practice and shows that there is a very short learning curve. Another limitation is the follow-up of eight weeks after surgery. Therefore, long-term follow-up data is not available to evaluate long-term complications such as lymphedema.

The strengths of this study are the multicenter design, prospective nature and the randomization within a patient. Randomization within one patient results in the reduction of many patient-related potential confounders. In addition, another strength is the standardization of both the surgical procedure and postoperative drainage of the groin.

In conclusion, we have demonstrated that LigaSure is feasible for IFL and shows promising results in terms of reduced postoperative morbidity compared to conservative IFL. Validation in a large cohort is needed to implement this new technique in clinical practice.

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The efficacy of ultrasound in the follow-up after a negative sentinel lymph node in vulvar cancer patients: a prospective single center study

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ABSTRACT

Objective To determine the efficacy of the addition of an ultrasound of the groins in routine follow-up of patients with vulvar squamous cell carcinoma (SCC) after a negative sentinel lymph node (SLN).

Design Prospective single-center study.

Setting A tertiary expert oncology center for the treatment of vulvar cancer.

Population All vulvar SCC patients with a negative SLN, treated between 2006 and 2014.

Methods We prospectively collected data of 139 vulvar SCC patients treated with an SLN procedure. We analyzed data of 76 patients with a negative SLN. Three-monthly follow-up visits consisted of physical examination combined with an ultrasound of the groins by a radiologist.

Main outcome measures The diagnostic value of ultrasound in the follow-up of vulvar SCC patients with a negative SLN during the first two years after treatment.

Results During a routine visit, two asymptomatic isolated groin recurrences were detected. Both patients were treated by inguinofemoral lymphadenectomy and adjuvant radiotherapy, and are alive without evidence of disease 39 and 120 months after diagnosis. In total, 348 ultrasounds and 29 fine needle aspiration were performed. The sensitivity of ultrasound to detect a groin metastasis was 100% (95% CI 16%-100%) and specificity 92% (95% CI 89%-95%).

Conclusions Routine follow-up including ultrasound of the groin led to early detection of asymptomatic isolated groin recurrences. Further research is necessary to determine the exact role of ultrasound in the follow-up of vulvar SCC patients with a negative SLN.

INTRODUCTION

The sentinel lymph node (SLN) procedure has been integrated as standard care for a subgroup of patients with early stage vulvar squamous cell carcinoma (SCC) after the publication of the GROINSS-V-I and GOG-173 study [1, 2]. If the SLN contains metastatic tumor, a inguinofemoral lymphadenectomy (IFL) is indicated. In patients with a negative SLN, close follow-up is advised with the primary aim to identify locoregional recurrences as early as possible.

The largest long term follow-up study of 377 patients with an SLN for primary vulvar SCC, showed isolated groin recurrences in 2.3% of the SLN negative patients [3]. In smaller, mainly retrospective, studies the isolated groin recurrence rate varied between zero and 6.6% [4, 5].

The main purpose of close follow-up in patients with a negative SLN was to detect groin recurrence in asymptomatic patients as early as possible to achieve better survival, preferably with limited morbidity. As lymph node palpation is difficult to interpret for small nodes, especially in people who are overweight, the question rises whether an ultrasound of the groins during follow-up has additional value for early detection of a groin recurrence.

Until now, there is no literature evaluating the additional value of ultrasound in the follow-up of vulvar SCC patients with a negative SLN. The current guidelines for follow-up after the SLN procedure in vulvar SCC are based on expert opinions rather than evidence. The Dutch guideline and The European Society of Gynaecological Oncology (ESGO) recommend follow-up in the first two years, including clinical examination of the groins but do not mention routine use of imaging of the groins [6, 7]. Furthermore, the Royal College of Obstetricians and Gynaecologists (RGOC) states that recognition of recurrence as early as possible seems logical because salvage is largely dependent on either further excision or radiotherapy. However, there are no recommendations on performing an ultrasound or not [8]. This implies the need for more knowledge with respect to the efficacy of an ultrasound of the groin in the follow-up after a negative SLN in vulvar SCC patients.

The primary aim of this study was to determine the diagnostic value of ultrasound in the follow-up of vulvar SCC patients with a negative SLN during the first two years after treatment. Furthermore, our secondary aims were to determine: the adherence to the follow-up protocol and to estimate the costs of performing ultrasound for the detection of a recurrence.

METHODS

A prospective study was performed at the Radboud university medical center, which is an expert center for the treatment of vulvar cancer. In 2006, with the knowledge of the preliminary results of the GROINSS-V-I study (2-3% groin recurrences with poor prognosis) combined with the evidence for an ultrasound in the preoperative assessment of the lymph node status, we decided to add ultrasound of the groin to the routine follow-up of patients with a negative SLN. We prospectively stored data of patients treated with a SLN from 2006 until the moment of closure (end of 2016) of the GROINSS-V-II study (NCT01500512) in an electronic database.

We selected patients if they met all of the following inclusion criteria: 1) primary unifocal macro-invasive vulvar SCC with a diameter of < 4 cm cm and clinically non-suspicious groin lymph nodes by palpation and without abnormality at ultrasound and/or fine needle aspiration cytology (FNAC), 2) SLN treatment between 2006 and 2014 and 3) unilateral negative SLN after a unilateral procedure or bilateral negative SLN after a bilateral procedure.

Patients were not engaged in the development of our study. The local ethical committee of the Radboud university medical centre approved this study (reference number 2017-3191). There was no funding for this study.

A unilateral SLN procedure was performed in patients with lateralized tumors (≥1cm from midline) and ipsilateral lymph flow on lymphoscintigraphy; in all other patients a bilateral SLN procedure was performed. The procedure was performed using the combined technique (radioactive tracer and blue dye) to identify the SLNs as described previously [9]. The removed SLNs were histopathologically examined by an expert gynecologic pathologist, including ultrastaging if necessary [1]. Patients with a (unilateral) positive SLN(s) had an indication for further therapy of both groins.

Routine follow-up consisted of a three-monthly visit at the outpatient clinic within the first two years after the SLN procedure. These visits included gynecologic examination with palpation of the groins by the gynecologist and a routine ultrasound by the radiologist. In patients with a unilateral SLN procedure, both groins were examined during follow-up. For logistical reasons, either the palpation or the ultrasound was performed first. After the first two years, follow-up is less frequent (twice a year) without the performance of a routine ultrasound of the groin.

The ultrasound of the groin was performed by our ultrasound unit at the Department of Radiology. This is an expert center with a dedicated team of professionals for the performance of ultrasound for different types of cancer.

Suspicious lymph nodes on ultrasound were characterized by a short-axis diameter ≥10 mm in oval shaped lymph nodes or ≥8 mm in circular shaped lymph nodes with a malignant aspect. Malignant aspects visualized by ultrasound are hilar hypoechogenicity, general attenuation, irregularity of the margin or abnormal vascular pattern

on Doppler (see Figure 3A-C). If a suspicious lymph node for metastasis was present, FNAC was performed in the same session.
An isolated groin recurrence was defined as a histologically proven metastasis of vulvar SCC in a lymph node in the groin, without a simultaneous local recurrence. In all patients with an isolated groin recurrence, we reviewed the indication for the SLN procedure and the technical aspects of the procedure itself, and an expert pathologist reviewed the pathological slides of the SLN(s).

Cost analysis

In our medical center, the cost of performing an ultrasound by the radiologist applies €75.-, independent of inspecting one or both groins. A FNAC costs €329.-, including histopathologic examination.

Statistical analysis

The start of the routine follow-up was defined as the date of the SLN procedure. All routine and indicated visits during the follow-up period starting at 2.5 months and going to 25.5 months after the SLN procedure were included for analyses. Not all visits took place exactly at the planned time. In the analysis we assigned the data to the nearest targeted visit moment, using a time window of three months; visits occurring from 1.5 months before the planned moment of visit till 1.5 months after the planned moment of visit, i.e. all results from the period between 4.5 and 7.5 months are analyzed as the 6-month visit. Only the result of the first visit, planned at month 3, is based on a shorter time window, as data were included from 2.5 months till 4.5 months after the SLN procedure.
The duration of total follow-up was defined as date of the SLN procedure until date of last follow-up or death.
The sensitivity, specificity, positive predictive and negative predictive value of the ultrasound and FNAC were calculated, and the Clopper-Pearson exact method was used to determine the 95% confidence intervals [10]. The test characteristics of palpation of the groin was not the aim of this study. A positive ultrasound (the presence of suspicious lymph nodes) and a positive FNAC was considered as truly positive if histopathologic examination of the lymph nodes retrieved by IFL showed metastatic disease. A negative ultrasound or FNAC was considered as truly negative if there was no evidence of metastatic disease in the groin after at least one year of follow-up.
Analyses were performed with SPSS version 22.0 [11].

RESULTS

Patient characteristics

As shown in Figure 1, 139 patients underwent an SLN procedure between 2006 and 2014. We excluded 49 patients because of a unilateral or bilateral positive SLN, two patients because definitive histopathologic examination showed basal cell carcinoma, and another 12 patients were excluded because they had a multifocal/larger tumor, a technical inadequate SLN procedure, or were lost to follow-up. In conclusion, we analyzed data of 76 patients with a negative SLN.

The median age was 67 years (range 37-89) and the median body mass index was 25.6 kg/m^2 (range 16.5 – 36.1). A bilateral SLN procedure was performed in 58 patients; a unilateral procedure was performed in 18 patients. The median number of dissected nodes was 3 (range 1-7) per patient after a bilateral SLN procedure and 2 (range 1-4) after an unilateral procedure. Median total follow-up time was 47 months (range 3-142). For patient- and tumor characteristics, see Table 1.

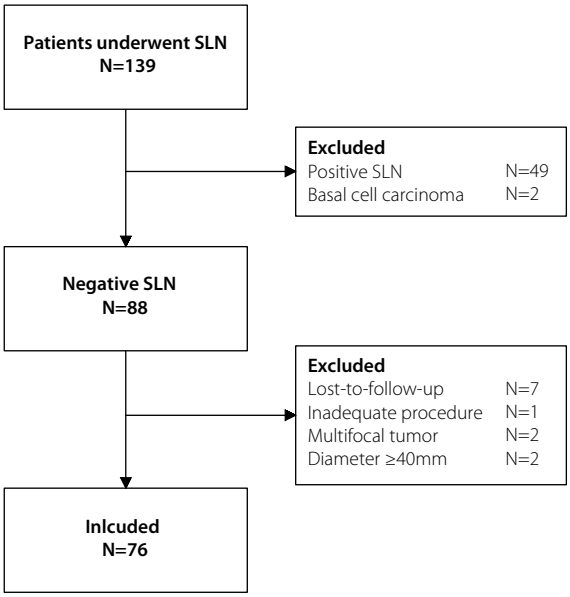


Figure 1 Study flow diagram

Footnote: SLN: sentinel lymph node, N: number of patients

Table 1 Patient- and tumor characteristics

Patient- and tumor characteristics	Median (range)	N (%)
Age (years)	67 (37-89)	
Body mass index (kg/m²)	25.5 (16.5-36.1)	
FIGO stage (2009)		
- IA		1 (1)
- IB		75 (99)
Localization		
- central		58 (76)
- lateral (≥1 cm from midline)		18 (24)
Tumor diameter (mm)	14.5 (1.0-40.0)	
Depth of invasion (mm)	3.0 (1.0-13.2)	
Grade of differentiation		
- I		25 (33)
- II		43 (57)
- III		8 (10)
Number of dissected sentinel nodes per patient	3 (1-7)	
- Bilateral procedure	2 (1-4)	
- Unilateral procedure		

Protocol adherence

Based on a schedule of 3-monthly routine visits during 2 years, the planned number of routine visits would be 608 (76 x 8). Due to shorter follow-up of patients who died or for whom vulvar cancer recurrence was detected, the expected number of planned routine visits was 529. In total, 408 (77% of expected) follow-up visits were performed, of which 403 were routine and five were interval visits because of complaints. Figure 2 shows the expected number of visits and the performed number of visits with and without an ultrasound per time period after the SLN procedure.

During the first year, 225/279 (81%) of the expected visits were performed compared with 183/250 (73%) in the second year.

The median number of performed follow-up visits was 7 (range 0-8) per patient. Four patients did not show up at any follow-up visit for different reasons: one patient had a local recurrence within three months after the SLN procedure, two patients chose not to participate, and in one patient the reason is unknown.

As shown in Table 2, in 348 of the 408 (85%) visits an ultrasound was performed; 29 (8%) ultrasounds showed one or more suspicious lymph nodes in the groin(s). As a consequence an FNAC was performed of all suspected lymph nodes. In one patient, cytologic examination was inconclusive; however, there was no evidence of a groin recurrence during a follow-up at 92 months and therefore the FNAC was considered as

negative. In two patients, cytologic examination of the FNAC showed a groin recurrence (see Isolated groin recurrences). In both patients, an ultrasound was performed during a routine follow-up visit and a suspicious lymph node was identified in the groin, and a subsequent FNAC was performed. However, in one patient there was a suspected lymph node in the groins at palpation. All ultrasound and/or FNAC negative patients were followed for at least one additional year and did not show a groin recurrence.

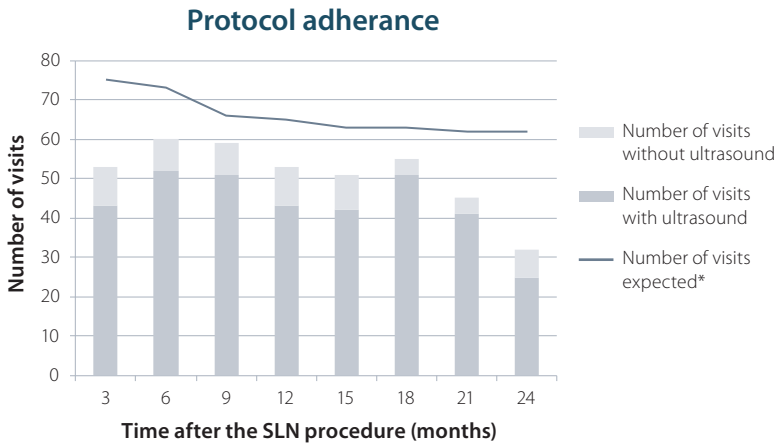


Figure 2 Protocol adherence

Footnote:*number of expected visits was corrected for the actual follow-up time of each patient by taking into consideration the date of death or detection of a vulvar cancer recurrence.

Isolated groin recurrences

Using ultrasound, isolated groin recurrence of the vulvar SCC was diagnosed in two of 76 patients (2.6%); both recurrences were diagnosed within eight months after the SLN procedure during a routine follow-up visit, see Table 3. Groin recurrence did not occur in any of the other patients. recurrence occurred.

The patient (see Table 3, patient A) was diagnosed with a lateralized tumor (≥ 1 cm from midline) on the left side of the vulva, during clinical examination and an ultrasound of both groins, there was no suspicious lymph node identified and a unilateral SLN procedure was performed; the lymphoscintigram showed ipsilateral lymph flow and two SLNs were identified and removed. The SLN procedure was adequate and no substandard factors could be identified afterwards. The patient went for a routine follow-up visit four months after treatment, she had no complaints and no abnormal lymph nodes were palpated by the gynecologist. However, ultrasound showed one

Table 2 Summary of results of palpation, ultrasound and FNAC

Time after SLN procedure in months	Number of visits	Number of patients with suspicious groins by palpation	Number of patients in which ultrasound was performed	Number of patients with suspicious lymph nodes on ultrasound	Number of patients in which FNAC was performed	Number of patients with positive cytology*
3	53	2[a]	43	6[d]	6[d]	1
6	60	3[b]	52	4[e]	4[e]	1
19	59	3[b]	51	7[e]	7[e]	0
12	53	4[c]	43	3[d]	3[d]	0
15	51	1[a]	42	2	2	0
18	55	3[c]	51	4	4	0
21	45	2[a]	41	2	2	0
24	32	1[a]	25	1	1	0
Total	**408**	**19**	**348**	**29**	**29**	**2**

Footnote: FNAC: fine needle aspiration cytology, SLN: sentinel lymph node, [a]in all patients also suspicious on ultrasound, [b] in one patient not suspicious on ultrasound, [c] in two patients not suspicious on ultrasound, [d]in two patients bilateral, [e] in one patient bilateral, *for more detailed information see Table 3.

suspicious lymph node in the contralateral groin (see Figure 3B) and FNAC was performed; cytologic examination showed SCC. Treatment consisted of bilateral IFL. Histopathologic examination showed five lymph nodes in the left side without evidence of disease, and eight lymph nodes in the right groin; in one lymph node there was a metastasis of the known vulvar SCC, with a diameter of seven millimeters without extranodal growth. Cloquet's node from both the left and right did not contain metastatic disease. Adjuvant radiotherapy was given. This patient is alive without evidence of disease 120 months after the diagnosis of the groin recurrence.

The patient (See Table 3, patient B) was diagnosed with a midline tumor, without suspicious groin lymph nodes by palpation and ultrasound, and was treated by a bilateral SLN procedure; in total three SLNs were identified and removed. The procedure was adequate and no substandard factors could be identified afterwards. However, eight months later, this patient visited the outpatient clinic for routine follow-up. The gynecologist identified a suspicious lymph node in the right groin at palpation and subsequently an ultrasound of both groins was performed. This ultrasound showed one suspicious lymph node in the right groin (see Figure 3C) and one suspicious lymph node in the left groin; a FNAC of both lymph nodes was performed. Cytologic

Table 3 Characteristics of patients with an isolated groin recurrence

Patient characteristics	Patient A	Patient B
Age (years)	60	77
BMI (kg/m2)	27.1	23.5
Primary tumor		
Tumor localization	Lateral, left	Midline
FIGO stage	IB	IB
Tumor diameter (mm)	5	20
Depth of invasion (mm)	4.3	8.0
Grade of differentiation	I	II
Primary treatment		
Procedure adequate after revision	Yes	Yes
Dissected SLNs - Left - Right	 2 -	 1 2
Isolated groin recurrence		
Groin recurrence	Right	Right
Time to recurrence (months)	3.8	7.4
Moment of diagnosis	Routine visit	Routine visit
Complaints	No	No
Palpable suspicious lymph nodes	No	Yes, right
Number of lymph node metastases	1	1
Diameter lymph node metastasis (mm)	7	34
Extranodal growth	No	No
Treatment		
Bilateral inguinofemoral lymphadenectomy - Number of lymph nodes removed right (number of lymph nodes with metastatic disease) - Number of lymph nodes removed left (number of lymph nodes with metastatic disease)	 9 (1) 6 (0)	 17 (1) 11 (0)
Adjuvant radiotherapy	Yes	Yes
Survival		
Patient status	Alive without evidence of disease (120 months)	Alive without evidence of disease (39 months)

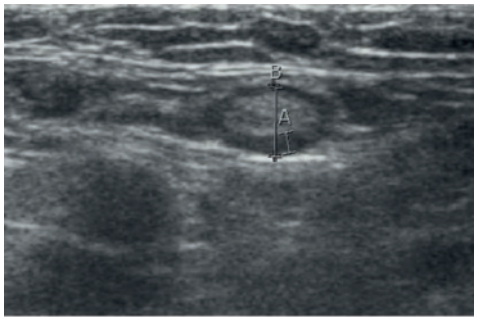
examination showed malignant cells fitted with SCC in the right groin, no evidence of malignant disease was seen in the cytologic material of the left groin.
Bilateral IFL was performed and histopathologic examination showed 17 lymph nodes from the right groin, with one lymph node metastasis measuring 34 millimeters without extranodal growth. Examination of the removed tissue of the left groin showed 11 lymph nodes, without metastatic disease. Adjuvant radiotherapy was performed and the patient is without evidence of disease 39 months after the groin recurrence.

Test characteristics

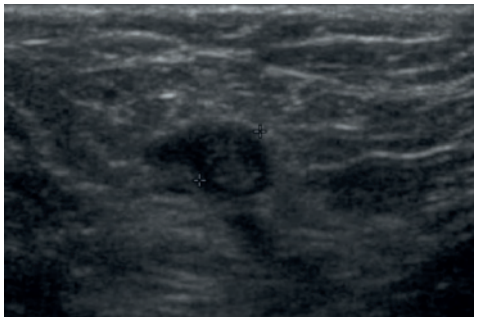
Test characteristics of the ultrasound in the follow-up were as follows: the sensitivity of ultrasound to detect a groin metastasis was 100% (2/2; 95% CI 16-100) , the specificity 92% (319/346; 95% CI 89-95), positive predictive value (PPV) 6.8% (2/29; 95% CI 0.9-23) and negative predictive value (NPV) 100% (346/346; 95% CI 99-100).
For paired ultrasound and FNAC (FNAC after a positive ultrasound), the sensitivity was 100% (2/2; 95% CI 16-100), specificity 100% (27/27; 95% CI 87-100) as well as PPV and NPV were 100% (PPV: 2/2; 95% CI 16-100), NPV: 27/27; 95% CI 87-100).

Costs

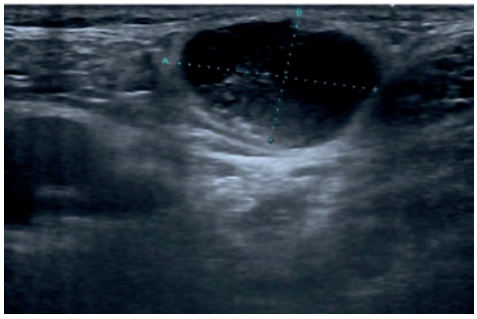
In this study, one isolated nonpalpable groin recurrence was identified by the routine ultrasound of the groin. The other groin recurrence would have been detected by a follow-up regimen without a routine ultrasound of the groin, as this recurrence was palpable during clinical examination. The detection of the non-palpable asymptomatic groin recurrence required 348 ultrasounds (€26 100) and a FNAC in 29 patients (€9 541), generating total costs of €35 641. This results in additional costs of €469 per patient.



A Ultrasound image of a normal lymph node in the groin (measurement A: 1.4 mm, B: 4.6 mm)



B Ultrasound image of a metastatic lymph node (patient A): enlarged (diameter 5.9 mm), focal cortical thicking on the left side and loss of echogenic hilar sinus fat



C Ultrasound image of a metastatic lymph node (patient B): enlarged oval shaped, loss of echogenic hilar sinus fat (measurement A: 23.1 mm, B: 14.3 mm)

Figure 3 Ultrasound images

DISCUSSION

Main findings

We evaluated the diagnostic value of ultrasound of the groins in the follow-up of 76 vulvar SCC patients after a negative SLN. Routine ultrasound in the follow-up during the first two years resulted in the early diagnosis of one asymptomatic, nonpalpable groin recurrence. In our study, 348 ultrasounds and a FNAC in 29 patients were necessary to detect this recurrence. The sensitivity of ultrasound to detect a groin metastasis was 100% (95% CI 16-100) and specificity 92% (95% CI 89-95).

Strengths and limitations

Our prospective study is the first determining the efficacy of ultrasound in the follow-up of patients after a negative SLN. In addition to obvious limitations such as low number of patients and low number of events, the single center design is a possible drawback. Furthermore, the ultrasounds were performed by different radiologists working in our specialized ultrasound unit; this may have introduced bias because ultrasound examination and interpretation of the ultrasound features as the indication for a metastatic lymph node are radiologist dependent. However, this reflects daily clinical practice. Furthermore, the cost analyses were performed with the costs of an ultrasound and FNAC at our Dutch medical center and therefore may not be reproducible for all medical centers in other countries.

Interpretation

A groin recurrence in vulvar SCC patients is nearly always fatal; published literature reports a 5-year survival of only 0-10% [12-15]. More recently, a 7-year survival of 50% was reported in one study [16]; this retrospective study described all 30 patients treated for a groin recurrence (isolated or a groin and pelvic recurrence). Primary groin treatment could be either an IFL or an SLN. Follow-up consisted of clinical examination and, if needed, imaging of the groin.

The reported seven-year survival of 50% is much higher than the previously reported 0-10% [12-16]. This might imply that patients with a groin recurrence after a negative SLN procedure, may have a better prognosis compared with patients with a groin recurrence after an IFL. This difference may be explained by either the (multimodal) treatment options available and/or pathogenesis. An isolated groin recurrence after an IFL can be caused by in-transit metastases in lymph channels, as all lymph nodes are removed. On the contrary, an isolated groin recurrence after an SLN is more likely to be the result of remaining (isolated) tumor cells in the lymph nodes, probably with less spread outside the lymph node. However, all patients with a groin recurrence after a negative SLN in the GROINSS-V-I study, in whom follow-up only consisted of palpation of the groin, died of disease [3]. In contrast, in our study both patients with a groin recurrence

are still alive. Therefore, early detection and resection of a groin recurrence, for example by routine ultrasound may be of importance for improved survival.
In penile SCC patients, the effect of early versus late resection of lymph node metastases was evaluated [17]. This study retrospectively selected 40 patients with clinically node negative groin lymph node metastases treated with lymphadenectomy. The first 20 patients were treated with a 'wait and see' policy and strict follow-up; when the lymph node(s) became clinically apparent and metastases were cytologically proven, lymphadenectomy. The other 20 patients were treated using the SLN procedure, and if a positive SLN was present, an additional lymphadenectomy. The disease specific 3-year survival of the first group was 35% versus 84% for the second group ($p = 0.0017$). These results underline the importance of early diagnosis and resection of groin lymph node metastases.Besides, the SLN procedure is also common practice in breast cancer patients. In these patients, the axillary recurrence rate after a negative SLN is only 0.3% [18]. This is much lower compared with vulvar SCC and might be explained by differences in treatment; breast cancer patients more often receive (neo)adjuvant chemotherapy after a negative SLN. Furthermore, Leikola *et al.* concluded that, due to the low incidence of axillary recurrence, routine ultrasound of the axilla after a negative SLN was unlikely to be cost-effective[19]. We diagnosed two isolated groin recurrences within eight months after the SLN procedure; one might question if the lymph node metastases have been missed in preoperative workup and/or the SLN was false negative. In depth analyses of the indication for this SLN procedure, the pre-operative workup and the procedure itself, revealed no substandard factors. Therefore, these isolated groin recurrences may be the result of a false negative SLN, in which (isolated) tumor cells have not been identified.
In our study, both patients with a groin recurrence were asymptomatic, and remarkably, both patients are still alive without evidence of disease. Since the number of patients with a groin recurrence in our study was small, we were not able to calculate quality-adjusted life years (QALY) and perform a cost-effectiveness analysis. However, the ultrasound in patient A detected a nonpalpable groin recurrence, and this patient is still alive ten years after diagnosis. The costs for the ultrasounds and subsequent FNACs were €35 641. Given the fact that patient A survived at least ten years after diagnosis of the groin recurrence, the accepted threshold (in the Netherlands) of €50,000 per QALY is by far not reached.
To minimize the possible burden for the patient of a false positive ultrasound and the subsequent 'unnecessary' FNAC, ultrasound features that most accurately identify groin lymph node metastasis should be identified. In patients with breast cancer, ultrasound of the lymph nodes in the axilla to identify metastatic lymph nodes is frequently performed. In these patients, cortical thickness (with a 3 mm cut-off) is shown to be the most reliable ultrasound feature predicting a lymph node metastasis [20-22]. This characteristic is not routinely used in the evaluation of groin lymph nodes in patients with vulvar cancer but might be an option.

Future follow-up schedules should be personalized and risk-based; this can be based on body mass index, as this patient-related factor can negatively influence the detection of a (small) groin recurrence by palpation. More research is needed to identify patients at high risk for a groin recurrence to offer a personalized risk-based follow-up.
The best way to evaluate the value of using ultrasound in terms of better survival is a randomized controlled trial comparing a follow-up protocol with and without routine ultrasound. However, such a trial is not realistic given the low incidence of vulvar SCC on the one hand, and the limited number of groin recurrences on the other. The most suitable design is a prospective multicenter phase II study. Furthermore, with current concerns over rising healthcare costs, further evaluation is needed to determine the most cost-effective evidence-based follow-up schedule for patients with a negative SLN.

Conclusion

The performance of a routine ultrasound in the follow-up of 76 vulvar SCC patients with negative SLNs resulted in the early diagnosis of one nonpalpable groin recurrence, this patient is still alive without evidence of disease ten years after diagnosis. In our study, 348 ultrasounds and 29 FNACs were performed to detect this recurrence. In our view, this is counterbalanced by the earliest possible detection of groin recurrences leading to the best possible survival for the individual patient.

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10

General discussion

IMPROVING LOCAL TREATMENT

There are indications for a different biological behavior of human papilloma virus (HPV) related and non HPV-related vulvar squamous cell carcinoma (SCC); HPV-related carcinomas are smaller with less invasion depth, less frequently associated with groin lymph node metastases and comes with a better prognosis compared to non-HPV related carcinomas [1, 2]. Currently, there is no difference in treatment for both disease entities. The pathogenesis for the HPV-related vulvar SCC resembles the oncogenesis of the premalignancy cervical intraepithelial neoplasia (CIN) into cervical cancer. In HPV-related vulvar SCC, high grade squamous intraepithelial lesion (HSIL) is the precursor, which has a causal relationship with HPV in nearly 100% of the lesions. Among HPV-related vulvar SCC and HSIL, HPV 16 is the predominant type [3]. Substantial number of these lesions may be avoided by prophylactic vaccination against HPV and might decrease the incidence of HPV-related vulvar SCC in the future. In contrast, prophylactic vaccination will not affect the incidence of non HPV-related vulvar SCC, which are the majority of all vulvar SCCs. For the future, the main challenge is to understand pathogenesis of non-HPV related vulvar SCC.

DVIN is the precursor of non human papilloma virus related vulvar SCC

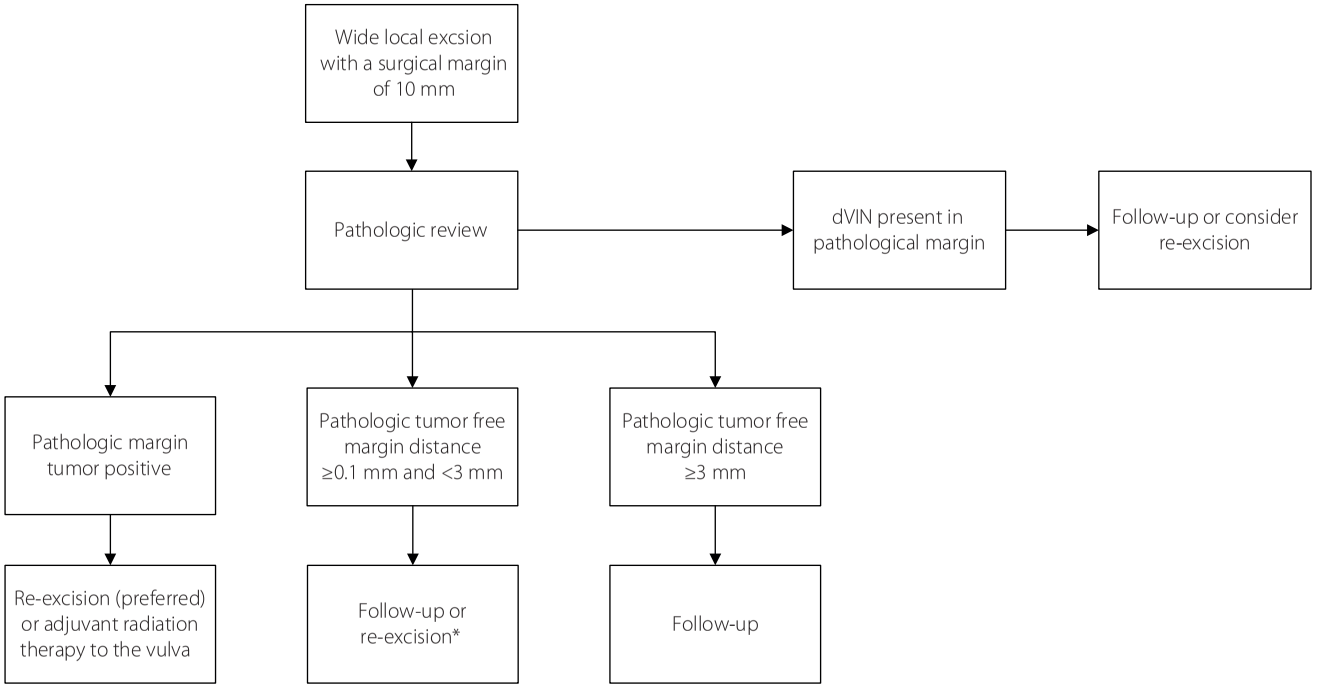
Non HPV-related vulvar SCC mainly arises in a background of the chronic inflammatory vulvar skin disease lichen sclerosus (LS) and/or differentiated vulvar intraepithelial neoplasia (dVIN). Because of the rarity of dVIN outside the setting of invasive vulvar SCC, the question raised whether dVIN should be considered as a true precursor lesion [4]. In Chapter 2 we provide genetic evidence that vulvar SCC originate from single precursor cells of LS and/or dVIN in which a subset of the genetic alterations detected in the carcinoma, possibly driving premalignant events, were already present in the precursor lesion. We identified copy number alterations in all vulvar SCCs, and in one patient three copy number alterations were shared between the vulvar SCC and the paired dVIN lesion. Besides, we identified identical *TP53* mutations in five vulvar SCCs and the paired dVIN lesion and in one patient also in the paired LS lesion. This genetic evidence for dVIN and/or LS as precursor lesion for vulvar SCC is supported by a recent study which showed shared somatic mutations in *TP53*, *NOTCH1* and *HRAS* between dVIN, LS and vulvar SCC [5].

Given the genetic evidence that dVIN is a precursor lesion of vulvar SCC, it is important to recognize dVIN by the clinician and the pathologist at an early phase. However, dVIN is a difficult diagnosis, both clinically and histopathologically. This contributes to a considerable underdiagnosis of (solitary) dVIN lesions; a study showed that 42% of the biopsies diagnosed as LS taken from patients who progressed to vulvar SCC, were classified as dVIN after histopathologic review by an expert gynecopathologist [6]. Attempts were made to improve the histopathologic recognition of dVIN lesions by

formulating diagnostic criteria to identify dVIN. Van den Einden *et al.* [7] formulated guidelines consisting of histopathologic characteristics of dVIN, and showed that agreement between pathologists slightly increased after studying these guidelines, especially among gynecopathologists.
In contrast to the high malignant potential of dVIN of around 33% [8], patients with LS have a relatively lower lifetime risk for vulvar SCC of around 4-7% [9, 10]. It is generally accepted that all patients with LS may have a life-long indication for yearly follow-up to early detect dVIN and/or vulvar SCC. A deeper understanding of the carcinogenesis of non HPV-related vulvar SCC might lead to a better identification of a specific subgroup of patients with LS at high-risk for progression to vulvar SCC, contributing to more personalized care.

Surgical margins

The recommended surgical tumor-free margin distance varies between different guidelines, ranging between one and two centimeters [11-14]. Tissue shrinkage after excision in cervical and head and neck carcinoma is reported to be around 10-15%, due to formalin fixation and/or intrinsic tissue properties [15, 16]. Worldwide a pathologic tumor free margin of ≥8 mm has been advocated as safe to minimize the risk for local recurrence. However, surgery to reach this margin can be mutilating, especially when the tumor is located close to important midline structures such as the clitoris, urethra and anus. As a consequence, smaller margins might result in less treatment related morbidity. The pathologic tumor margin distance with a cut-off value of 8 mm has frequently been challenged as a prognostic factor and is an increasing topic of debate. In Chapter 4 and 5 we show that patients treated for primary vulvar SCC have an ongoing risk for local recurrent disease, with a local recurrence rate as high as 43% within 10 years after treatment. The local recurrence rate was not associated with a pathologic tumor free margin of less than 8, 5 or 3 mm, but with dVIN in the pathological margin. In our cohort, in which a surgical margin distance of >10 mm was pursued, approximately 7% of the patients had a tumor positive margin and 8% a tumor free margin between 0 and 3 mm at primary excision. We recommend an intended surgical tumor free margin of 10 mm, but not to excise unnecessary tissue close to important midline structures such as the clitoris (see flowchart Figure 1). The results of our study should lead to implementation of a pathologic tumor free margin distance of ≥3 mm.
Ideally, a prospective randomized controlled trial comparing the differences in local recurrence rates between patients with a tumor free margin of < 8 mm with follow-up versus adjuvant therapy can provide the highest level of evidence regarding the question of whether a tumor fee margin of < 8 mm leads to a higher local recurrence rate. Based on the current evidence, it is not ethical in our opinion to expose patients with a tumor free margin between 3 and 8 mm to potential harmful adjuvant therapy such as re-excision and/or radiotherapy.



*If re-excision is possible in relation to important structures.

Figure 1

Some previous studies reported more local recurrences in patients with a pathologic tumor free margin distance < 8 mm compared to a wider margin. In our study, the local recurrence rate was not associated with a pathologic tumor free margin of less than 8 mm. A possible explanation for this contradictory finding might be the fact that in patients with a tumor free margin ≥ 8 mm the precursor lesion is more often excised with a clear margin. However, none of these studies reported data regarding the presence of a precursor lesion in the surgical margin. We showed in Chapter 5 that the presence of dVIN or dVIN in combination with LS in the margin resulted in a significantly higher local recurrence rate compared to patients without any precursor lesion in the margin. In our study, the presence of dVIN with or without LS was equally distributed between the groups of patients with a pathological margin < 8 mm versus ≥ 8 mm.
Local recurrences occur in 43% of the patients within ten years after treatment and have a negative impact on the prognosis [17]. A local recurrence can develop from either remaining tumor cells called a 'true' recurrence, or a 'de novo' tumor from a precursor lesion that was also the source of the primary tumor. The second can be explained by the concept of 'field cancerization', as first introduced by Slaughter *et al.* [18] in head and neck SCC: these genetically altered fields surround the tumor and can have a diameter of over seven centimeters [19]. A retrospective study including 13 patients with local recurrent head and neck SCC shows that in 5 cases (39%) a precursor lesion was absent and the primary and recurrent SCC had genetic similarity, providing evidence that residual cancer cells were the origin of recurrence. For the remaining eight cases (61%) a genetically related field was detected, and for five of these cases, evidence was found that both the primary and recurrent carcinoma originated from this field [20]. In addition, we showed a clonal relationship between vulvar SCC and the precursor lesions dVIN and LS in Chapter 2. In contrast to dVIN which are solitary lesions, LS is a field with large dimensions as it may affect the skin of the whole vulva.
The fact that the local recurrence rate is still 30% in patients without a precursor lesion in the margin might be explained by a premalignant field outside the margins, or early malignant changes in the margin not visible by histopathologic examination. Knowledge on molecular markers representing early malignant changes in the histopathologically normal epithelium might be helpful to identify the presence of such a cancerized field. In addition, molecular techniques might be an option for a more accurate risk assessment of these fields, to select patients with a high risk field for progression to a 'de novo' tumor. A drawback of assessing the margins to identify the presence of a cancerized field is the fact that the borders of a surgical excision specimen are large and not all surgical margins are included in the tissue blocks for histopathological examination. In addition, there might be a difference in the lateral and the basal resection margins in relation to the presence of a cancerized field. There is no literature concerning this. However, logically the lateral margins are more likely to contain a precursor lesion or the cancerized field rather than the basal margin.

An important clinical implication of the concept of field cancerization is that the cancerized field often remains after wide local excision of the primary tumor, and the risk for a 'de novo' tumor is relatively high for these patients. This hypothesis is supported by the higher local recurrence rate after wide local excision compared to radical vulvectomy, as summarized in Chapter 3.
LS, itself a very itchy condition, contributes to a vicious cycle of itching and scratching which may lead to superimposed lichens simplex, squamous cell hyperplasia and ultimately vulvar SCC; the itch-scratch hypothesis [21]. This hypothesis is supported by a recent prospective cohort study including 507 women with histologically proved LS, with the aim to evaluate the effect of preventive treatment with topical corticosteroid on the disease symptoms and the risk for dVIN and/or vulvar SCC [22]. In patients compliant to the prescribed topical corticosteroid treatment, there were less LS-related symptoms (itching, pain and dyspareunia) and less progression of adhesion or scarring, and a significantly lower incidence of dVIN and/or vulvar SCC compared to non-compliant patients.
In conclusion, treatment with topical corticosteroids in patients with LS may reduce the risk for developing vulvar carcinoma. Treatment of the cancerized field might reduce the risk of progression towards a 'de novo' tumor. However, surgery is not a real option because the large dimensions, in combination with the fact that it might not be clinically visible. Topical application might be an option and can be applied to the whole field.

IMPROVING GROIN TREATMENT

Determination of the inguinofemoral lymph node status by inguinofemoral lymphadenectomy (IFL) plays an important role in the treatment of vulvar SCC. This procedure is finally indicated in around half of the patients as part of treatment for primary (multifocal disease or tumor > 4 cm) or local recurrent vulvar SCC (if IFL was no part of primary treatment). Unfortunately, IFL has significant short- and long-term complications, which are reported in up to 85% of the patients [23-25]. In Chapter 7, we show that volume-controlled drainage resulted in significantly less complications compared to short drainage of the groin, and should be considered as standard of care. However, morbidity remains high and therefore new treatment strategies to replace IFL with less treatment-related morbidity but the same effectiveness and safety will be a big step forward for vulvar SCC patients. We will discuss how a reduction of the number IFLs can be achieved, taking in account accurate prediction of the presence of inguinofemoral lymph node metastases and new treatment strategies to replace IFL. In the second part, we will discuss how morbidity associated with IFL might be further reduced.

Prediction of inguinofemoral lymph node metastases

A reduction of the number of patients with an indication for groin surgery can be achieved by adequate prediction of the presence of lymph node metastases. Around 70% of the patients undergoing a SLN procedure has no lymph node metastases [26]. These patients will not benefit from the SLN procedure in terms of improved survival and do not have the benefits of reduced morbidity of the SLN anymore at time of local recurrence. On the other hand, not detecting a groin lymph node metastasis results in a very poor survival. Ideally, there is a non-invasive test which can safely exclude lymph node metastases with a high negative predictive value.

Until now, the depth of invasion is the only predictive histopathological factor in the staging and for the presence of lymph node metastases; patients with vulvar SCC with a depth of invasion of ≤1 mm (microinvasive growth) have a negligible risk for inguinofemoral lymph node metastases of less than 1%, and are therefore excluded from groin surgery. The depth of invasion is measured as recommended by the FIGO: measured from the epithelial-stromal junction of the most superficial adjacent dermal papillae to the deepest point of invasion. However, in many carcinomas such as cervical cancer, the depth of invasion is measured from the nearest dysplastic crypt or surface epithelium [27], because tumor cells will originate from the nearest rete ridges instead of the most superficial dysplastic epithelium. In vulvar SCC, this alternative measurement method was recently introduced and studied by Van den Einden *et al.* [28]. They showed that the alternative method to measure the depth of invasion might provide a better reflection of the prognosis. In addition, it results in microinvasive instead of macroinvasive growth in 19% of the patients, in turn resulting in a lower number of patients with an indication for groin surgery [28]. However, in one patient with a carcinoma assessed as microinvasive by the alternative method, there was a micrometastasis present in the groin. To determine the exact prognostic value of the depth of invasion classified by the alternative measurement method, further studies with a higher number of patients are needed in which the depth of invasion is measured using both methods. Such a study can give the answer of whether the alternative method gives a better reflection of the biological behavior of the carcinoma, in terms of presence or absence of lymph node metastases. Besides, the optimal cutoff for determining a group of patients with a negligible risk for lymph node metastases should be defined for the alternative method, as this might differ from the cut-off of 1 mm for the conservative method.

As the depth of invasion guides the need for groin surgery, reproducibility of these measurements is important. We showed in Chapter 3 that there was similar agreement in classifying the depth of invasion for both measurement methods. Between pathologists, there was slightly higher overall agreement using the alternative method. In addition, the ease of use was scored similar for both methods by the pathologists. This implies that the alternative method is suitable for pathologists to measure and

classify the depth of invasion in vulvar SCC. However, the question whether this alternative method reflects the prognosis should be answered first.
Besides the depth of invasion no other histopathologic parameters are known to define such a low risk group for inguinofemoral lymph node metastases in patients with vulvar SCC that inguinofemoral lymph node staging can be safely omitted. Better understanding of the driving force behind tumor growth by molecular and/or genetic analyses might lead towards better prediction of the presence of inguinofemoral lymph node metastases.

Replace IFL by new treatment strategies

Alternative treatment regimens instead of IFL for patients with a positive SLN are currently studied. The GROINSS-VII study (NCT01500512) aims to a assess the safety of replacing IFL by radiotherapy in patients with a micrometastasis (‹2 mm) in the sentinel lymph node. Inclusions are completed and results are expected. The GROINSS study group will continue to study the safety of chemoradiation for macrometastases (›2 mm) in the SLN.
Nowadays, the SLN technique is implemented for a selected group of patients with primary vulvar SCC. There will be a substantial advantage in terms of reduced morbidity if the SLN can also be performed in patients with local recurrent disease, since a high number of patients will develop local recurrent disease. The repeat SLN procedure for vulvar SCC patients with local recurrent disease is technically feasible, but the accuracy of this procedure has not been investigated in vulvar SCC yet [29]. A meta-analysis including breast cancer patients showed that in patients with local recurrent disease a repeat SLN procedure is safe if the SLN is negative at time of primary carcinoma [30]. To investigate the safety of repeat SLN procedure in recurrent vulvar SCC, the incidence of inguinofemoral lymph node metastases at time of local recurrence should be determined first. These data are needed to optimally counsel patients and should serve as a basis for a prospective multicenter study to answer the question of whether a repeat SLN in patients with local recurrent vulvar SCC is a safe alternative for IFL.

Reducing morbidity associated with IFL

In previous years, minimally invasive techniques are developed to reduce postoperative morbidity of inguinofemoral lymphadenectomy and promising results are published. However, one study reported complication rates after minimally invasive IFL [31] which are even higher compared to the complication rate in our studies after open IFL. In addition, the oncologic safety of this new approach is unclear. A prospective study with a minimum of two years follow-up, can answer the question regarding the oncologic safety of minimally invasive IFL.
In laparoscopic surgery electrothermal bipolar vessel sealing devices are used, in contrast to sharp knife dissection and/or bipolar diathermy in open IFL. One might

question if the minimally invasive procedure itself or the device used during this procedure are key for reducing morbidity. To investigate the role of the sealing device LigaSure in reducing the postoperative morbidity, we designed the prospective MAMBO-IC study (Chapter 8) to assess the differences in the incidence of short-term complications (wound infection, wound breakdown and lymphocele) using LigaSure versus the conventional performance of IFL randomized within each individual patient. We included 20 patients and showed that LigaSure for IFL is feasible and results in significantly lower estimated incidence of ≥1 complication per groin compared to conservative IFL. Patients' reported restriction of daily living activities and maximum pain score were scored equal for both treatment methods. There were no notable differences in the surgeons' reported workload scores. Future research, preferably a nationwide randomized controlled trial in a large cohort, comparing LigaSure versus conservative IFL is needed to validate our findings regarding the difference in post-operative morbidity between both surgical methods.

IMPROVING FOLLOW-UP

In patients treated for vulvar SCC, routine follow-up is performed with the primary aim to identify locoregional recurrences as early as possible. In this thesis, we focused on both local and groin recurrences.

In Chapter 4 and 5, we show that patients have an ongoing risk for local recurrence. In these studies we were not able to identify a subgroup of patients not at risk for local recurrence of vulvar SCC, but patients with dVIN in the surgical margin are at higher risk for local recurrent disease. Furthermore, the aim of routine follow-up should also be to detect a new precursor lesion as early as possible, to prevent progression to vulvar SCC. There is a lack of knowledge on the incidence and time to a new precursor lesion after the diagnosis and treatment of vulvar SCC. Future research should identify these important questions and thereafter, the follow-up regimen may be further individualized. In addition, identification of high-risk patients may further improve patient empowerment, patient education and may also contribute to individualized follow-up schedules. The (early) detection of a local recurrence might be improved by self-examination by the patient and/or her partner.

Besides detecting local disease, follow-up is performed to detect regional recurrent disease. The main purpose of close follow up in patients with a negative SLN is to detect groin recurrence in asymptomatic patients as early as possible to achieve better survival, preferably with limited morbidity. In Chapter 9 we showed that routine follow-up including an ultrasound of the groin led to early detection of two asymptomatic isolated groin recurrences, of which one was non-palpable.

One might question whether this close follow-up is indicated as a groin recurrence is considered as palliative. However, in our study both patients are still alive 39 and 120 months after diagnosis, and this finding is supported by Frey *et al.*, reporting a 7-years survival of 50% in patients with groin recurrence after a SLN or IFL. This supports the hypothesis that patients with a groin recurrence after a SLN might have a better prognosis compared to a groin recurrence after IFL. Future research should focus on in-depth analyses and survival of patients with groin recurrence after a SLN.

FUTURE PERSPECTIVES

Based on the immune microenvironment and/or somatic mutations, we might identify different etiologic pathways within the group of non HPV-related vulvar SCC. Identifying different subtypes has only additional clinical value if it reflects different tumor behavior and/or facilitates tumor-based treatment strategies.
A many studied immunosuppressive mechanism of cancer cells is by programmed cell death protein 1 (PD-1) and its ligand PD-L1 . There are few publications regarding PD-L1 expression in vulvar SCC and its precursor lesions. A study including 103 vulvar SCCs reported that PD-L1 is expressed in 10% and was more often present in non-HPV related vulvar SCC. In addition, PD-L1 expression was associated with significantly lower recurrence-free survival, both in univariate and multivariate Cox regression analyses [32]. Another study showed PD-L1 expression in 32% (27/84) of the vulvar SCC and significantly more often observed in p16-negative carcinomas [33]. PD-L1 expression was rare among HSIL (n=13) and dVIN (n=2) lesions; one HSIL lesion adjacent to a HPV-related carcinoma showed PD-L1 expression [34]. As PD-L1 is predominantly expressed in non HPV-related vulvar SCC and associated with a worse prognosis, PD-L1 blockade might improve prognosis in this subset of patients. There are no studies about therapeutic use of PD/PD-L1 blockade in vulvar SCC. Future research should identify the tumor immune microenvironment including PD-L1 expression in order to distinguish different tumor subtypes and to explore opportunities for immunotherapeutic treatment options for vulvar SCC.

Recently, based on the assessment of molecular alterations a third etiologic pathway of vulvar SCC was suggested. Within the group of non HPV-related vulvar SCC, there were two different subtypes identified based on somatic mutations: in around two-third a TP53 mutation was present and in one-third no *TP53* mutation was detected but a high frequency of *NOTCH1* and *HRAS* mutations [5]. In this study, targeted next generation sequencing (NGS) was performed of 36 vulvar SCC and revealed somatic mutations in 89% of the carcinomas and was equally distributed between HPV-related and non HPV-related vulvar SCC. *TP53* mutations more frequently in non HPV-related

compared to HPV-related vulvar SCC, 28% and 65% respectively. Besides *TP53*, *NOTCH1* and *HRAS* were frequently detected as mutation, in 33% and 28% of the carcinomas respectively. A *NOTCH1* mutation was only detected in non HPV-related vulvar SCC, and co-occurred with *TP53* mutations but was also identified in carcinomas that did not carry a *TP53* mutation. Zieba *et al.* [35] performed NGS including hotspot mutations in 50 genes on 81 vulvar SCC, of which 64% was HPV-related and 36% non HPV-related vulvar SCC. A pathogenic mutation was present in 61% of the carcinomas. The most frequently identified mutations were TP53 and CDKN2A, with frequencies equally distributed between HPV-related and non HPV-related vulvar SCC.

Driver mutations enable outgrowth of cancerous cell populations. It can be challenging to differentiate cancer-driving mutations from passenger mutations [36, 37]. In addition, cancer genotype is increasingly recognized as only part of the puzzle, and other aspects such as the tumor microenvironment might be equally important in determining behavior of tumor cells. Combined interpretation of cancer genotype and phenotype, including histology, might better characterize an individual tumor.

The signaling pathway activation status is determined not only by errors in the cancer cell (epi-) genome, but to a large extent by interactions between the cancer cell and its microenvironment [38]. Currently, ten to twelve cellular signal transduction pathways are known, relatively independent of the cancer cell type of origin. Knowledge of these cellular signal pathways on cancer cell behavior is rapidly growing, but it has not been evaluated in vulvar SCC yet.

Based on understanding of the driving force behind tumor growth it might be possible to predict tumor behavior, especially predicting spread to the inguinofemoral lymph nodes. In addition, it might identify new targets for personalized and tumor based treatment strategies.

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Summary | Samenvatting

SUMMARY

In this thesis, we describe our studies with the aim to optimize treatment and follow-up of patients with vulvar squamous cell carcinoma (SCC). **Chapter 1** introduces the pathogenesis, treatment and follow-up of vulvar SCC and outlines the research aims of this thesis.

Differentiated vulvar intraepithelial neoplasia (dVIN) is mostly found in a background of lichen sclerosus (LS) and can give rise to non HPV-related vulvar SCC, but genetic evidence is currently still lacking. In **Chapter 2**, we aimed to study the genetic relationship between LS, dVIN and vulvar SCC. We compared genomic abnormalities of vulvar SCC and patient-matched dVIN and LS lesions of twelve patients using genome-wide copy number profiling and targeted sequencing of *TP53*. High-resolution genome-wide copy number analysis was performed on six vulvar SCCs and three paired dVIN samples, and identified copy number alterations in all vulvar SCCs. In one patient, three copy number alterations were shared between the vulvar SCC and the dVIN lesion. Subsequent targeted sequencing of *TP53* in eight vulvar SCC samples identified mutations in five vulvar SCCs. All these five mutations were traced back in the paired dVIN lesions and in one paired LS lesion with frequencies of the mutation ranged from 3 to 19%, suggesting that mosaic subpopulations of cells in the LS and dVIN lesions gave rise to the vulvar SCC. In conclusion, our data provide genetic evidence for a clonal relationship between the precursor lesions LS, dVIN and non HPV-related vulvar SCC.

The depth of invasion is an important prognostic factor for patients with vulvar SCC and guides the need for groin surgery. In patients with a microinvasive SCC (depth of invasion ≤1 mm) treatment consists of wide local excision of the vulvar tumor only, while in patients with a macroinvasive SCC (depth of invasion >1 mm) a sentinel lymph node procedure and/or a full inguinofemoral lymphadenectomy is indicated. There are several methods described to measure the depth of invasion. The conventional standard method for vulvar SCC is recommended by the International Federation of Gynecology and Obstetrics (FIGO). This methods measures the distance between the epithelial-stromal junction of the most superficial adjacent dermal papillae and the deepest point of invasion. In carcinomas from other sites such as cervical cancer, the depth of invasion is measured in a different way. The depth of invasion is measured from the basement membrane of the deepest adjacent tumor-free rete ridge to the deepest point of invasion. This alternative method might give a better reflection of the prognosis. As the depth of invasion guides treatment, a high interobserver agreement in classifying the depth of invasion is crucial. In **Chapter 3**, we aimed to study the interobserver agreement for classifying the depth of invasion into ≤ 1 mm or > 1 mm for both measurement methods, to identify pitfalls and to formulate recommendations. Fifty slides with vulvar SCC with a depth of invasion around 1 mm were assessed by ten

pathologists and four pathologists in training. The depth of invasion was measured by each participant using both methods. We showed moderate agreement (kappa 0.57 (95%CI 0.45-0.68)) between pathologists classifying the depth of invasion using the conventional measurement method and similar agreement using the alternative method. We identified six pitfalls in assessing the depth of invasion: disagreement which invasive nest is deepest, recognition of invasive growth and where it starts, curved surface, carcinoma situated on the edge of the tissue block, ulceration and different methods used to determine the depth of invasion. We propose the following recommendations: optimizing and standardizing of sampling, measuring the least favorable depth of invasion if different locations are present, measuring from the floor of the tumor ulcer, and using the conventional method as standard method. In conclusion, we showed moderate agreement in assessing the depth of invasion using the conventional method, without a notable difference using the alternative method. This study showed that the alternative method is suitable for pathologists and already in use. However, before implementing this method in daily clinical practice, future research should be performed to determine if the alternative method leads to a better reflection of the prognosis, and of whether a new threshold needs te be defined to reflect biological tumor behavior.

Local recurrence of vulvar SCC is an important clinical issue; in patients with a local recurrence the disease-specific survival decreases significantly from 90% to 69% and all patients have an indication for inguinofemoral lymphadenectomy (IFL) if not performed as part of treatment for the primary vulvar SCC. The most debated prognostic factor for local recurrence is the tumor free margin. Currently, the recommended surgical tumor-free margin distance varies between different guidelines ranging between one to two centimeters, and a pathologic tumor fee margin of ≥8 mm has been advocated as safe. However, surgery to reach this margin can be mutilating, especially if the tumor is close to important midline structures such as the clitoris, urethra and anus. Knowledge on prognostic factors related to local recurrences should be helpful to select high risk patients and/or to develop strategies to prevent local recurrences. In Chapter 4, we summarized the current knowledge on the incidence of local recurrences related to clinicopathologic and cellbiologic variables. We indicated an estimated local recurrence rate of 4% per year without plateauing. The prognostic relevance for local recurrence of vulvar SCC of all analyzed variables remains equivocal, including pathologic tumor free margin distance <8 mm, presence of lichen sclerosus, groin lymph node metastases and a variety of primary tumor characteristics (grade of differentiation, tumor size, tumor focality, depth of invasion, lymphovascular space invasion, tumor localization and presence of human papillomavirus). We concluded that the current evidence on prognostic factors for local recurrence of vulvar cancer is not sufficiently robust to allow evidence-based clinical decision making.

In Chapter 5, we present our multicenter study to determine the incidence of local recurrence of vulvar SCC in relation to tumor and/or precursor lesion free pathologic margins. This study was performed in a clinically well-defined consecutive patients series (N=287) of two oncology centers with pathology review. We show that patients treated for primary vulvar SCC have an ongoing risk for local recurrent disease, and the local recurrence rate is as high as 43% within 10 years after treatment. The results of our study showed that a pathologic tumor free margin distance of less than 8, 5 or 3 mm was not associated with a higher local recurrence rate compared to a wider tumor free margin. Multivariable analyses showed a higher local recurrence rate in patients with dVIN and LS in the margin, in patients with dVIN in the margin, and a FIGO stage II or higher. Based on these results, we propose to change the current guidelines and to implement a intended surgical tumor free margin of 10 mm, but also not to excise unnecessary tissue close to important midline structures. Besides, we propose to lower the cut-off for a save pathological tumor free margin distance to ≥ 3 mm, and to consider re-excision if dVIN is present in the pathologic margin. As our study showed that patients have an ongoing risk for local recurrence and we were not able to identify a subgroup of patients not at risk for local recurrence, we recommend to continue life-long follow-up for all patients.

Determination of the inguinofemoral lymph node status by IFL plays an important role in the treatment of vulvar SCC. Eventually, this procedure is indicated in around half of the patients with vulvar SCC, either at time of primary disease or at time of local recurrent disease. IFL has significant short- and long-term complications, which are reported in up to 85% of the patients. In Chapter 6, we created an up-to-date summary of the modifications of the surgical techniques and peri- and postoperative care protocols which were studied in order to reduce the treatment associated morbidity. We concluded that after the implementation of several adjustments of the surgical techniques the morbidity after IFL decreased but remains high and clinically meaningful. In Chapter 7, we aimed to assess the feasibility and the incidence of short-term complications after volume-controlled versus short drainage of the groin; the MAMBO-IA and IB studies (Morbidity And Measurement of the Body). In this nationwide prospective study, we included 77 patients for volume-controlled and 64 patients for short drainage. Both treatment protocols were feasible but the volume-controlled protocol was more difficult to follow: in around half of the groins the drain was removed in deviation of the protocol due to wound complications, drain problems or logistic difficulties. Volume-controlled drainage was associated with significantly less lymphoceles compared to short drainage, without a difference in the incidence of wound infection or primary wound dehiscence. The estimated incidence of one or more complications per groin was 46% after volume-controlled drainage versus 75% after short drainage, (RD 29% (95% CI 8, 49) p= 0.006). We therefore recommend volume-controlled drainage as the standard of care.

Nevertheless, after volume-controlled drainage complications are still present in a significant proportion of the groins. Adaptations in the surgical approach may play a key role in reducing morbidity associated with IFL. In Chapter 8, we aimed to study the feasibility of LigaSure for IFL and to assess the incidence of short-term complications, using LigaSure versus the conventional performance of IFL. Moreover, we aimed to evaluate patients' and surgeons' experience for both surgical methods. In this MAMBO-IC study we randomized in 20 patients for which groin the LigaSure was used, for the other groin the conventional IFL was performed. To reduce patient-related potential confounders, we randomized within each individual patient. The incidence of a wound infection, primary wound dehiscence and lymphocele per groin did not differ between the two surgical treatment methods. The estimated incidence of ≥1 complication(s) per groin was 29% after LigaSure versus 70% after conventional IFL (risk difference 41% (95% CI 19-62), $p < 0.001$). Patients' reported restriction of daily living activities and maximum pain score were scored equal for both treatment methods. There were no differences in the surgeons' reported workload scores. In conclusion, LigaSure is feasible and possibly associated with significantly less short-term complications compared to conventional IFL. Validation in a large cohort is needed before implementing this new technique in clinical practice.

Isolated groin recurrences after a negative sentinel lymph node (SLN) occur in 2-3% of the patients. The consequences of a groin recurrence are significant, with a five year survival of 0-10%. After a negative SLN close follow-up is performed to identify locoregional recurrences as early as possible to achieve better survival rates, preferably with limited morbidity. In Chapter 9 we aimed to determine the efficacy of the addition of an ultrasound of the groins in the routine follow-up of patients with vulvar SCC after a negative SLN. This study included 76 patients undergoing three-monthly follow-up visits which consisted of physical examination combined with an ultrasound of the groins by a radiologist. Two asymptomatic isolated groin recurrences were detected by ultrasound, of which one was non-palpable. The sensitivity of ultrasound to detect a groin metastasis was 100% (95% CI 16%-100%) and specificity 92% (95% CI 89%-95%). Both patients with an isolated groin recurrence were treated by inguinofemoral lymphadenectomy and adjuvant radiotherapy and are alive without evidence of disease 39 and 120 months after diagnosis. In total, 348 ultrasounds and 29 fine needle aspiration cytology procedures were necessary to detect these two recurrences. In our view, this is counterbalanced by the earliest possible detection of groin recurrences leading to the best possible survival for the individual patient. Future research should focus on developing personalized and risk-based follow-up schedules; this might be based on body mass index, as this patient-related factor can negatively influence the detection of a (small) groin recurrence by palpation. Besides, future research should focus on the survival after a groin recurrence, as patients with a groin recurrence after a (negative) SLN procedure

may have a better prognosis compared with patients with a groin recurrence after an IFL.
Finally, in **Chapter 10** the content of this thesis is discussed in a broader sense. Furthermore, we provide clinical implications and possible directions for future research.

SAMENVATTING

In dit proefschrift beschrijven wij onze studies met het doel om de behandeling en follow-up van patiënten met een plaveiselcelcarcinoom van de vulva te optimaliseren. In **hoofdstuk 1** geven wij een overzicht van de pathogenese, behandeling en follow-up van het plaveiselcelcarcinoom van de vulva, tezamen met onze onderzoeksdoelen en de hoofdlijnen van dit proefschrift.

Gedifferentieerde vulvaire intraepitheliale neoplasie (dVIN) wordt vaak gezien in een achtergrond van de huidziekte lichen sclerosus (LS) en kan ontaarden in een plaveiselcelcarcinoom van de vulva. Dit type vulvacarcinoom wordt niet veroorzaakt door het humaan papillomavirus (HPV). Echter, genetisch bewijs dat dVIN en LS een voorloperstadium zijn van het plaveiselcelcarcinoom ontbreekt. Het doel van **hoofdstuk 2** is om de genetische relatie van zowel LS als dVIN met het niet-HPV-gerelateerde plaveiselcelcarcinoom te bestuderen. In deze studie hebben wij van twaalf patiënten het DNA van het plaveiselcelcarcinoom vergeleken met het DNA van de LS en dVIN. Dit hebben wij gedaan met behulp van genomisch analyse waarbij wij ons hebben gericht op veranderingen in kopie aantallen van chromosomen en chromosoom segmenten, en gerichte en zeer gevoelige sequentie bepaling van het gen *TP53*. De genoombrede genoom analyse van de plaveiselcelcarcinomen heeft geleid tot het vaststellen van afwijkingen in genomische kopie aantallen in alle plaveiselcelcarcinomen. In het weefsel van één patiënt waren enkele genomische afwijkingen zoals gevonden in het plaveiselcelcarcinoom identiek aanwezig in de dVIN-laesie. Daarnaast vonden wij in vijf plaveiselcelcarcinomen *TP53* mutaties, die ook aanwezig waren in de dVIN-laesie van eenzelfde patiënt en in LS van één patiënt met mutatie frequenties variërend tussen de 3 en 19%. Deze data bewijzen dat er een genetische relatie is tussen enerzijds de voorlopers LS en dVIN en anderzijds het niet-HPV-gerelateerde plaveiselcelcarcinoom van de vulva.

De invasiediepte is een belangrijke prognostische factor voor patiënten met een plaveiselcelcarcinoom van de vulva en speelt een heel belangrijke rol bij de keuze van de behandeling. Indien de invasiediepte ≤ 1 mm is, bestaat de behandeling uit ruime lokale excisie van de vulvaire tumor. Indien de invasiediepte > 1 mm is, wordt er liesklierchirurgie toegevoegd aan de behandeling bestaande uit hetzij een schildwachtklierprocedure dan wel een liesklierdissectie. De invasiediepte kan op verschillende manieren gemeten worden. Door de International Federation of Gynecology and Obstetrics (FIGO) wordt aanbevolen om te meten met behulp van de conservatieve methode: vanaf de meest oppervlakkige dermale papil tot het diepste punt van invasie. In verschillende andere carcinomen, waaronder het cervixcarcinoom, wordt de invasiediepte met de alternatieve methode gemeten. Deze methode meet van de diepste en meest dichtbij gelegen tumorvrije papil tot het diepste punt van invasie. Deze alternatieve methode reflecteert mogelijk beter de prognose van de individuele

patiënt. Gezien het feit dat de invasiediepte leidend is voor het al dan niet chirurgisch behandelen van de lies, is het cruciaal dat er een goede overeenstemming is tussen de pathologen bij het beoordelen van de invasiediepte. **Hoofdstuk 3** heeft tot doel om de interobserverovereenstemming te bepalen voor het indelen van de invasiediepte in ≤ 1 mm of › 1 mm voor beide meetmethodes. Het tweede doel van deze studie is om valkuilen te identificeren bij het bepalen van de invasiediepte, om vervolgens aanbevelingen te formuleren voor pathologen. Voor deze studie hebben wij 50 coupes met een plaveiselcelcarcinoom van de vulva geselecteerd waarin de invasiediepte rond de 1 mm is.In deze coupes is de invasiediepte met beide methoden gemeten door tien pathologen en vier pathologen in opleiding. Dit resulteerde in een matige overeenstemming (kappa 0.57 (95%CI 0.45-0.68)) tussen de pathologen indien de conservatieve methode is gebruikt en een vergelijkbare overeenstemming indien de alternatieve methode is gebruikt. Wij stelden zes valkuilen vast bij de beoordeling van de invasiediepte: verschil in de keuze van de diepste invasieve nest, herkenning van (het begin van) invasieve groei, onregelmatig oppervlakte, positie van het plaveiselcelcarcinoom aan de rand van het weefselblok, ulceratie, en het gebruik van verschillende meetmethodes. Wij hebben een aantal aanbevelingen: het insluiten van weefsel standaardiseren, de minst gunstige invasiediepte meten indien er verschillende mogelijkheden zijn, meten vanaf de bodem van het ulcus en om de conservatieve methode als standaard te gebruiken. Samenvattend laten wij een matige overeenstemming zien tussen pathologen bij het meten van de invasiediepte, zonder een opvallend verschil tussen beide meet- methodes. Onze studie laat zien dat de alternatieve methode bruikbaar is voor pathologen en ook al gebruikt wordt. Alvorens deze methode te implementeren in de dagelijkse klinische praktijk is er meer onderzoek nodig om te bepalen of deze methode inderdaad een optimale reflectie geeft van het biologische gedrag van de tumor.

Het lokaal recidief van het vulvaire plaveiselcelcarcinoom is een belangrijk klinisch probleem. De ziekte specifieke overleving daalt significant van 90% bij patiënten zonder lokaal recidief naar 69% bij patiënten met een lokaal recidief. Daarnaast hebben alle patiënten met een lokaal recidief een indicatie voor een liesklierdissectie indien deze niet is verricht ten tijde van de behandeling van het primaire plaveiselcelcarcinoom van de vulva. De meest bediscussieerde prognostische factor voor een lokaal recidief is de tumorvrije marge. De huidige richtlijnen adviseren een chirurgische tumorvrije marge wisselend tussen de één en twee centimeter, en een pathologische tumorvrije marge van ≥8 mm. Echter, de chirurgische ingreep om deze tumorvrije marges te halen kan erg mutilerend zijn, met name wanneer de tumor dichtbij belangrijke functionele structuren ligt zoals de clitoris, anus en urethra. Meer kennis over prognostische factoren voor een lokaal recidief zal leiden tot een betere herkenning van hoogrisicopatiënten en/of de ontwikkeling van preventieve behandelingen. In **hoofdstuk 4** geven wij een samenvatting van de huidige literatuur omtrent klinische,

pathologische en celbiologische factoren in relatie tot de incidentie van het lokaal recidief. Hierin laten wij een geschat lokaal recidiefcijfer zien van 4% per jaar, dat op gelijk niveau blijft in de tijd. Daarnaast is de prognostische waarde van alle geanalyseerde variabelen twijfelachtig, inclusief de tumorvrije marge van ‹ 8 mm, de aanwezigheid van lichen sclerosus, lymfekliermetastasen in de lies, en meerdere tumorkarakteristieken van de primaire tumor (differentiatiegraad, diameter, focaliteit, invasiediepte, lymphangioinvasie, lokalisatie en de aanwezigheid van HPV). Wij concluderen dat het bewijs in de huidige literatuur omtrent de prognostische factoren voor een lokaal recidief onvoldoende is om het huidige behandelbeleid aan te passen.

In **hoofdstuk 5** beschrijven wij de resultaten van onze studie met het doel de incidentie van het lokaal recidief te bepalen in relatie tot tumor- en voorloperstadiumvrije pathologische marges. In deze studie hebben wij een groep van 287 opeenvolgende patiënten geïncludeerd die zijn behandeld in een van de twee deelnemende centra (UMCG en Radboudumc). Van alle geïncludeerde patiënten zijn de coupes gereviseerd door twee expertgynaecopathologen. Wij laten zien dat het lokaal recidiefcijfer 43% is binnen tien jaar na behandeling van het primaire plaveiselcelcarcinoom. Daarnaast kunnen wij op basis van de resultaten van deze studie concluderen dat een tumorvrije marge van minder dan 3, 5 of 8 mm niet gerelateerd is aan meer lokale recidieven vergeleken met een marge boven deze afkappunten. Daarnaast laten multivariabele analyses zien dat er een hoger lokaal recidiefcijfer is bij patiënten met dVIN en LS in de marge, dVIN in de marge en/of een FIGO stadium II of hoger. Op basis van bovenstaande resultaten adviseren wij een chirurgische tumorvrije marge van 1 cm zonder onnodig weefsel te verwijderen van belangrijke functionele structuren zoals de urethra en/of clitoris. Tevens adviseren wij om een pathologische tumorvrije marge van ≥ 3 mm te implementeren en een re-exisie te overwegen indien dVIN aanwezig is in de pathologische marge. Gezien de aanhoudende kans voor een lokaal recidief gedurende de jaren na het primaire plaveiselcelcarcinoom van de vulva en er geen subgroep patiënten geïdentificeerd kon worden die niet at-risk zijn voor een lokaal recidief, adviseren wij het continueren van levenslange follow-up voor alle patiënten die zijn behandeld voor een plaveiselcelcarcinoom van de vulva.

Het bepalen van de aan- of afwezigheid van lymfekliermetastasen door middel van een liesklierdissectie heeft een belangrijke rol in de behandeling van patiënten met een plaveiselcelcarcinoom van de vulva. Een liesklierdissectie is uiteindelijk geïndiceerd in ongeveer de helft van de patiënten ten tijde van het primaire carcinoom of ten tijde van het lokaal recidief. Deze ingreep gaat gepaard met forse morbiditeit: korte- en/of langetermijncomplicaties zijn gerapporteerd tot bij 85% van de patiënten. In **hoofdstuk 6** hebben wij de huidige literatuur samengevat omtrent de aanpassingen van de chirurgische techniek van de liesklierdissectie en de peri- en postoperatieve zorg met het doel de morbiditeit van deze ingreep te verminderen. Wij concluderen dat na de aanpassing van de chirurgische techniek de morbiditeit enigszins is afgenomen,

maar nog steeds aanzienlijk is. In **hoofdstuk 7** beschrijven wij de resultaten van onze prospectieve landelijke achtereenvolgende MAMBO-IA/IB-studies (Morbidity And Measurement of the BOdy). Deze studies hebben tot doel de haalbaarheid en het verschil in de incidentie van postoperatieve complicaties te bepalen van twee verschillende drainageprotocollen van de lies: op basis van volume (volume-controlled) versus korte drainage gedurende vijf dagen. In deze studie zijn 77 patiënten geïncludeerd voor de volume-controlled drainage en 64 patiënten voor de korte drainage. Wij concludeerden dat beide drainageprotocollen haalbaar zijn, alhoewel het volume-controlled drainageprotocol lastiger uit te voeren is. Bij ongeveer de helft van de patiënten behandeld met volume-controlled drainage is de drain niet volgens het protocol verwijderd vanwege wond complicaties, drain problemen of om logistieke redenen. Volume-controlled drainage resulteerde in significant minder lymphoceles zonder een verschil in de incidentie van wondinfecties en/of het spontaan opengaan van de wond. Tevens was de geschatte incidentie van een of meer complicaties per lies significant lager na volume-controlled drainage in vergelijking tot korte drainage van de lies: 46% versus 75% (RD 29% (95% CI 8, 49) p= 0.006). Gebaseerd op onze onderzoeksresultaten raden wij volume-controlled drainage aan als standaard behandeling.
Alhoewel volume-controlled drainage leidt tot minder complicaties, zijn er nog steeds een fors aantal liezen waarin een complicatie optreedt. Aanpassingen aan de chirurgische techniek van de klierdissectie kunnen een belangrijke rol spelen in het verder verminderen van de incidentie van postoperatieve complicaties. In **hoofdstuk 8** bestuderen wij in de MAMBO-IC-studie de haalbaarheid van het gebruik van LigaSure voor de liesklierdissectie. Tevens vergelijken wij in deze studie de incidentie van korte-termijncomplicaties na het gebruik van LigaSure in vergelijking met de conservatieve liesklierdissectie met behulp van een mes en/of diathermie. Daarnaast heeft deze studie het doel de ervaring van zowel de patiënt als de operateur voor beide methodes te evalueren. In deze studie zijn twintig patiënten geïncludeerd die allen een dubbelzijdige liesklierdissectie hebben ondergaan. Voorafgaand aan de liesklierdissectie is er gerandomiseerd binnen een patiënt voor welke lies (links of rechts) de LigaSure gebruikt werd. In de andere lies is de conventionele methode gebruikt. Er is gekozen voor randomisatie binnen een patiënt om mogelijk verstorende patiëntfactoren te reduceren. Bij beide liezen werd de volume-controlled drainage uitgevoerd. De incidentie van een lymphocele, wondinfectie en het spontaan opengaan van de wond, verschilde niet significant tussen beide behandelmethoden. Wel was er een significant verschil tussen de geschatte incidentie van een of meer complicaties per lies, namelijk 29% na LigaSure versus 70% na conservatieve liesklierdissectie (risk difference 41% (95% CI 19-62), p < 0.001). Er in geen verschil in de door de patiënt gerapporteerde beperking in dagelijkse activiteiten en de maximale postoperatieve pijnscore, en de door de operateur gerapporteerde werklastscore. Wij concluderen dat LigaSure haalbaar is voor de liesklierdissectie en leidt tot een significant lager

geschatte incidentie van liezen met een of meer complicaties. Alvorens deze techniek te implementeren in de dagelijkse praktijk is validatie van onze resultaten in een groter cohort patiënten nodig.
Na een negatieve schildwachtklier als onderdeel van de behandeling van een primair plaveiselcelcarcinoom van de vulva, treden geïsoleerde liesrecidieven op bij 2-3% van de patiënten. Een geïsoleerd liesrecidief heeft een erg slechte prognose, met een vijfjaars overleving van 0-10%. Na een negatieve schildwachtklierprocedure ondergaan patiënten strikte follow-up om een lokaal of regionaal recidief zo vroeg mogelijk te diagnosticeren, met het doel om betere overlevingscijfers te bereiken met zo min mogelijk morbiditeit. In **hoofdstuk 9** evalueren wij de waarde van het toevoegen van een echo van de lies aan de follow-upbezoeken bij patiënten met een negatieve schildwachtklier. In deze studie includeerden wij 76 patiënten die driemaandelijks voor een follow-upbezoek kwamen en waarbij er lichamelijk onderzoek werd verricht in combinatie met een echo van beide liezen door een radioloog. Deze echo's van de lies resulteerden in de diagnose van een asymptomatisch geïsoleerd liesrecidief bij twee patiënten, waarvan bij één patiënt het recidief niet palpabel was. De sensitiviteit van de echo om een liesmetastase op te sporen was 100% (95% CI 16%-100%) en de specificiteit 92% (95% CI 89%-95%). Beide patiënten met een geïsoleerd liesrecidief zijn behandeld met een liesklierdissectie met adjuvante radiotherapie en zijn 39 en 120 maanden na diagnose in leven zonder tekenen van een nieuw liesrecidief. Er waren in totaal 348 echo's en 29 cytologische puncties nodig om deze twee recidieven op te sporen. Naar onze mening weegt dit op tegen de zo vroeg mogelijke diagnose van een liesrecidief en de best mogelijke overleving voor de individuele patiënt. Vervolgonderzoek moet uitwijzen of de follow-up voor patiënten met een negatieve schildwachtklier verder gepersonaliseerd kan worden gebaseerd op het risico voor een liesrecidief, bijvoorbeeld op basis van de body mass index (BMI). Een hoger BMI is een mogelijke factor is die het opsporen van een liesrecidief door middel van lichamelijk onderzoek kan belemmeren. Tevens zal vervolgonderzoek uit moeten wijzen of er een verschil is in de overleving tussen patiënten met een liesrecidief na behandeling met een (negatieve) schildwachtklierprocedure of na behandeling met een liesklierdissectie.
In het laatste hoofdstuk, **hoofdstuk 10**, worden op basis van de resultaten van bovengenoemde studies de klinische implicaties en het toekomstperspectief bediscussieerd.


A

Appendix

ABOUT THE AUTHOR

Anne-Floor Pouwer werd op 21 augustus 1990 geboren in Kesteren. Zij groeide op samen met haar twee oudere zussen en jongere broer. In 2008 haalde zij haar VWO-diploma aan het Ichthus College te Veenendaal. Aansluitend startte zij met de studie geneeskunde aan de Radboud Universiteit en verhuisde zij naar Nijmegen. Voorafgaand aan de Master geneeskunde, vertrok Anne-Floor in 2011 naar Nieuw-Zeeland voor een onderzoeksstage bij de Cochrane Menstrual Disorder and Subfertility Group aan de Auckland University te Auckland onder begeleiding van Prof. C. Farquhar (Auckland University) en Prof. dr. J.A.M. Kremer (Radboudumc). Deze onderzoeksstage resulteerde in haar eerste publicatie en maakte Anne-Floor enthousiast voor wetenschappelijk onderzoek. Anne-Floor sloot haar coschappen af met het Schakeljaar Verloskunde en Gynaecologie in het Rijnstate Ziekenhuis te Arnhem en het Radboudumc. In 2015 nam Anne-Floor haar artsendiploma in ontvangst, waarna zij startte als arts assistent niet in opleiding (ANIOS) aan de afdeling Verloskunde en Gynaecologie in het Rijnstate Ziekenhuis te Arnhem. Na klinische werkervaring op te hebben gedaan lonkte de wetenschap. In 2016 is zij begonnen aan een promotietraject over het vulvacarcinoom aan de afdeling Gynaecologische Oncologie van het Radboudumc onder begeleiding van Prof. dr. Leon Massuger (afdeling Gynaecologie), Dr. Joanne de Hullu (afdeling Gynaecologie), Dr. Hans Bulten (afdeling Pathologie) wat resulteerde in dit proefschrift. Anne-Floor is momenteel werkzaam op de intensive care in het Viecuri te Venlo en zal volgend jaar starten met de opleiding tot gynaecoloog in cluster Nijmegen. Anne-Floor woont samen met Ruud Draak in Nijmegen.

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Te Grootenhuis NC*, **Pouwer AW***, de Bock GH, Hollema H, Bulten J, van der Zee AGJ, de Hullu JA, Oonk MHM. Margin status revisited in vulvar squamous cell carcinoma. Gynecol Oncol. 2019 May 17. [Epub ahead of print]

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