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ETIOLOGIC, DIAGNOSTIC AND PROGNOSTIC FACTORS IN VULVAR CANCER

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Carpe Diem

To my family

ABSTRACT

Vulvar Squamous Cell Carcinoma (VSCC) can be divided into two subgroups basaloid and/or warty (HPV-associated) and keratinizing/non-keratinizing carcinomas (not HPV-associated).

The inguinal lymph node status represents the most powerful prognostic factor. The Sentinel Node (SN) procedure is an alternative to complete inguinofemoral lymphadenectomy, which diminishes the complications. In this thesis the value of preoperative lymphoscintigraphy and the SN procedure was assessed. It was discovered that the relation between SNs detected on the scintigram and those found during surgery showed good agreement using Weighted Kappa. The detection rate of SN was 98% for radioisotope plus blue dye, and 94% for blue dye alone. The false negative rate was 2.7%.
Hr-HPV (16, 18, 33, or 52) was detected in 31% of the tumours and in 43% of the SNs in patients with HPV-positive tumours. Patients with HPV-positive VSCC were significantly younger at diagnosis and had better survival. SNs with metastases were more frequently HPV-positive than those without metastases.
As in many other cancer forms, there is a need for new and better prognostic markers in vulvar cancers. High expression of ln-5γ2 chain and HPV negativity were associated with poor outcome. In a multivariate analysis only HPV status and tumour stage were significant factors for survival. Ln-5γ2 expression showed positively significant correlation with stage, tumour-size, grade and metastases, but was negatively associated with HPV status. Expression of the proliferation marker Ki-67 was significantly correlated with HPV status.
Studies of the proteome characteristics of HPV- positive versus HPV-negative VSCC by protein and pathway profiling on a global and individual tumour level detected four proteins as playing a major role in discriminating relapse from non-relapse tumours: STAT1, MX1, LGMN and PSMA5. Validation by immunohistochemistry showed significant down-regulation in HPV-positive compared to HPV-negative tumours. In the individual tumour pathway analysis, the pathways “RIG-1 like receptors in antiviral innate immunity” and “Rac signalling” emerge discriminate for separating relapse from non-relapse.

In conclusion; preoperative scintigram gives the best estimate of the accurate number of lymph nodes but cannot determine if unilateral or bilateral groins should be explored in cases of midline tumours. Presence of HPV DNA in SN was related to metastatic disease but did not affect survival.
High expression of ln-5y2 chain and HPV negativity were associated with poor outcome. However in multivariate analysis only HPV status and FIGO-stage showed significant relation to survival. Alterations of the “RIG-1 like receptors in antiviral innate immunity” pathway may be linked to an unfavourable prognosis, while alterations of the “IFN/EGFR/Glucocorticoid” signalling pathway is associated with HPV-positive tumours and thus of favourable prognosis.

Key words; Sentinel node biopsy, vulvar cancer, false negative rate, preoperative lymphoscintigraphy, HPV, laminin-5 γ2 chain, DNA ploidy, Ki-67, quantitative proteome profiling.

LIST OF PUBLICATIONS

I. Lindell G, Jonsson C, Ehrsson RJ, Jacobsson H, Gemzell-Danielsson K, Nordström KB, Larson B. **Evaluation of preoperative lymphoscintigraphy and sentinel node procedure in vulvar cancer.** *Eur J Obstet Gynecol Reprod Biol. 2010 Sep;152(1):91-5. Epub 2010 Jun 29*

II. Lindell G, Näsman A, Jonsson C, Ehrsson RJ, Jacobsson H, Gemzell-Danielsson K, Dalianis T, Nordström KB, Larson B **Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node** *Gynecol Oncol. 2010 May;117(2):312-6. Epub 2010 Feb 6*

III. Lindell G, Castro J, Näsman A, Gemzell-Danielsson K, Ehrsson RJ, Dalianis T, Barbro Larson B , Nordström K B **Laminin-5γ 2 chain expression, Nuclear DNA content and proliferative activity, as predictors of survival in vulvar cancer.** *Submitted*

IV. AnnSofi Sandberg, Gunnel Lindell, Rui Mamede Branca, Brita Nordström Källström, Barbro Larson, Kristina Gemzell-Danielsson, Jenny Forshed, Janne Lehtiö **Investigating the proteome characteristics of Human Papilloma Virus positive versus negative vulvar carcinoma by iTRAQ-based Mass Spectrometry – protein profiling on a global and individual tumour level.** *In manuscript*

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CONTENTS

LIST OF ABBREVIATIONS

2 DE	Two dimensional gel electroforesis
Aneuploidy	Cytometrically determined nuclear DNA content that deviates from that of normal cells
BM	Basal membrane
CT	Computer tomography
DAPI	4',6-diamidino-2-phenylindole
Diploidy	The DNA content (2c)corresponding to a diplod number of chromosomes (2n) in a normal human cell is 46
DNA	Deoxyribonucleic acid
E2F	Group of genes that codifies transcription factors
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ErbB	Protein family belonging to the EGFR group
ESI	Electrospray ionization
FADD	Fas-associated death domain
FCM	Flow cytometry
FDR	False discovery rate
FIGO	International Federation of Gynaecology and Obstetrics
FTICR	Fourier transform ion cyclotron resonance
GO	Gene ontology
GPCR	G protein-coupled receptors
H&E	Hematoxylin and Eosin staining
HER-2	Human epidermal growth factor receptor 2
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
Hr HPV	High risk human papilloma virus
IFN	Interferon
IHC	Immunohistochemistry
IMC	Image cytometry
iTRAQ	Isobaric tag for relative and absolute quantification
LC	Liquid chromatography
LGMN	Legumain protein
Ln	Laminin
LS	Lichen sclerosus
LVSI	Lympho Vascular Space Involvement
m/z	Mass to charge ratio
MALDI	Matrix assisted laser desoprtion ionisation
MAYU	Software package for the analysis of mass spectrometry-based proteomics data sets
MHC	Major histocompatibility complex, a gene family important for immune system
MIB-1	A monoclonal antibody that recognises the nuclear protein encoded by the Ki-67 gene, expressed in proliferating cells
MRI	Magnetic resonance image
MS	Mass spectrometry
MX1	Myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 protein

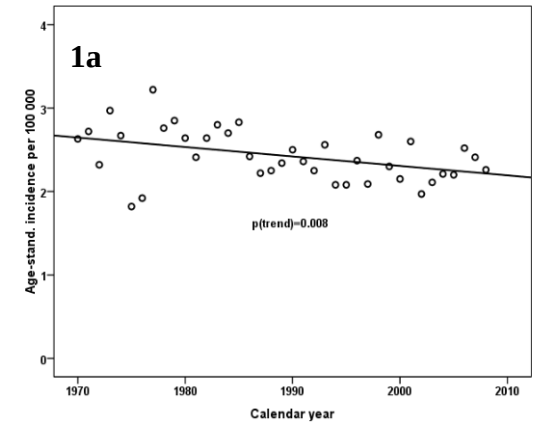
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
O-PLS	Orthogonal partial least square analysis
P14	Nuclear protein encoded by the p14 gene acting as a tumour suppressor
P16INK4A	Cyclin-dependent kinase inhibitors protein encoded by the p16 gene
P53	Nuclear protein encoded by the p53 gene acting as a tumour suppressor.
PCR	Polymerase chain reaction
PET	Positron emission tomography
p*I*	Isoelectric point
PQPQ	Software for quantification of mass spectrometry-based proteomics data sets
pRB	Retinoblastoma protein family
PSMA 5	Proteasome subunit alpha type-5 protein
Q	Quadropole
RCT	Randomized controlled trial
SCC	Squamous cell carcinoma
SN	Sentinel node
SPF	S-phase fraction
STAT 1	Signal transducer and activator of transcription 1 91kDa protein
TMA	Tissue micro array
TOF	Time-of-flight
VC	Vulvar cancer
WHO	World health organisation
VIN	Vulvar intraepithelial neoplasia
VSCC	Vulvar squamous cell carcinoma

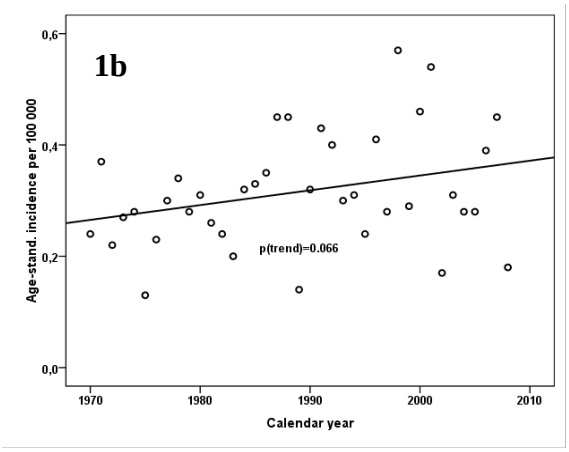
1 INTRODUCTION

1.1 BACKGROUND AND EPIDEMIOLOGY

Cancer in the female genital organs constitutes approximately 12% of all female cancer. [1]. Vulvar cancer (VC) accounts for about 4% (app. 140 cases/year) of all gynaecological cancers in Sweden and for about 3% of all gynaecological cancers in the US [2]. It mostly affects older women. Vulvar squamous Cell Carcinoma (VSCC) accounts histologically for 90% of vulvar cancers [3] and is, according to the WHO classification subdivided into basaloid, warty, verrucoid and keratinizing/non-keratinizing carcinomas [4-6]. Melanomas, adenocarcinomas, basal-cell carcinomas and sarcomas constitute the remainder. Dissemination of VSCC is predominantly lymphogenic to the inguinofemoral nodes. Distant metastases are rare. The overall survival rate is < 60%, (Cancer incidence in Sweden 2008). The incidence of VSCC varies, with the highest incidence in Europe and North America and the lowest in developing countries [2]. The differences may, to some extent, be explained by insufficient reporting of cases from the developing countries likely to be influenced by low life expectancy. Recent data indicates that invasive vulvar carcinoma, and especially its precursor lesions Vulvar Intraepithelial Neoplasia (VIN), have increased in incidence. According to a population-based study in Norway, the incidence of VIN has increased threefold between the periods 1973 to 1977 and 1988 to 1992 [7]. Another study from the US showed that vulvar carcinoma *in situ* has increased by 411% from 1973 to 2000 and invasive vulvar cancer by 20% [8]. Studies from the National Cancer Institute identified vulvar carcinoma as one of 12 cancers with an increase in incidence between 1992 and 1998; the increase has occurred predominantly among females younger than 65 [9]. In another study from the US an increase of VIN III by 3.5% per year and invasive cancer by 1% per year was reported [10]. In a European study patients treated for VC at the university hospital in Dusseldorf doubled in the last three decades and there was an almost fourfold increase in younger patients [11]. However, in a population based study from the Netherlands, the incidence of usual VIN and differentiated VIN increased between 1992 and 2005, while the incidence of VSCC remained stable [12].

In Sweden the age standardized incidence rate of VSCC shows a significant decrease depending on a significant decrease (p=0.002) among elderly women (50 to >85 years) while the incidence rate for women 0-49 years shows a slight increase (p=0.066) (figure 1a-c).





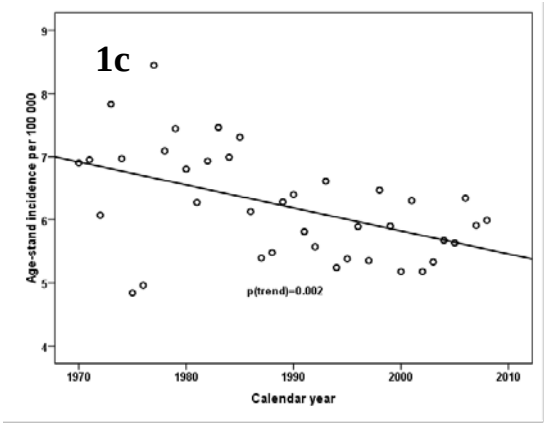


figure 1, *Age standardized incidence rate of Vulvar Squamous Cell carcinoma (VSCC) per 100 000 by calendar year* ***1a****;All women .* ***1b****; Women 0-49 years.* ***1c****; Women 50 to > 85 years. Standard population: the Swedish female population 2000 (data from cancer incidence in Sweden 2008) not previously published*

1.2 ETIOLOGIC FACTORS

The development of VSCC follows two separate pathways of which one is associated with HPV and the other is not [4-6]. Classic or usual VIN lesions (figure 2), showing a high rate of HPV infection are precursor of the HPV associated type. Histologically the carcinomas are non-keratinizing basaloid and/or warty (figure 2) and contain hr-HPV predominantly 16 and 18. Women with HPV-positive tumours are generally younger, show a high rate of multifocal disease and often associated cervical, vaginal and anal intraepithelial neoplasia**.** Tumours of the other pathway are keratinizing/non-keratinizing carcinomas, commonly HPV-negative, associated with differentiated VIN (figure 2) and lichen sclerosus (LS) [13-16]**.**

The proportion of HPV-positive VSCC varies from 9 to 70% in different studies [13, 17-23]. The proportion of HPV-positive VSCC in Sweden has not been previously investigated and although the presence of HPV has been examined in SN in cervical carcinoma and in lymph nodes in general in VSCC [20, 23], we have not found any studies on the presence of HPV in SN in VSCC.

1.2.1 The oncogenic pathway of HPV-positive VSCC

It is well established that hr-HPV´s are the cause of cervical carcinoma, of which 99% are HPV- positive [24, 25], and play a role in other malignancies including head and neck cancer, penile cancer and vulvar cancer.

The hr-HPV viruses exert their oncogenic capacity primarily via the viral proteins E6 and E7, which are the primary transforming viral proteins targeting two major cellular regulators, the retinoblastoma protein family (pRB) and p53. By blocking p53 and pRb controlled pathways, E6 and E7 keep infected cells in a proliferative state which is necessary for viral DNA synthesis [26]. Gene expression profiles of HPV-positive and HPV-negative cancers show differences in cell cycle genes with an up-regulation in HPV-positive tumours. Mediated by E2F activity, causing over-expression of E2F regulated genes [27].

E6 and E7 also act to prevent the antiviral and anti-tumour effects of the Interferon (IFN)-mediated immune response [28]. Thus by evading the immune system, the HPV infection is maintained.

1.2.2 The oncogenic pathway of HPV-negative VSCC

In contrast to HPV-positive VSCC, little is known about what pathways are deregulated in the HPV-negative VC [29-31]. It has been speculated whether an unknown viral aetiology (unidentified types of HPV or novel polyomaviruses) in fact causes those HPV-negative vulvar carcinomas [32]. HPV- negative cancer is associated with differentiated VIN and/or LS. Most of the differentiated VINs are associated with p53 mutations or deletions. Differentiated VINs and VSCC have been shown to share identical p53 mutations supporting a pathogenic connection [30]. The poorest disease-specific survival in vulvar carcinoma is seen in HPV-negative tumours with high p53 protein levels and low p14 (ARF) [21]. It is not known if in VSCC the same protein networks that are main drivers in HPV/non-HPV are also main drivers in relapse/non-relapse tumours.

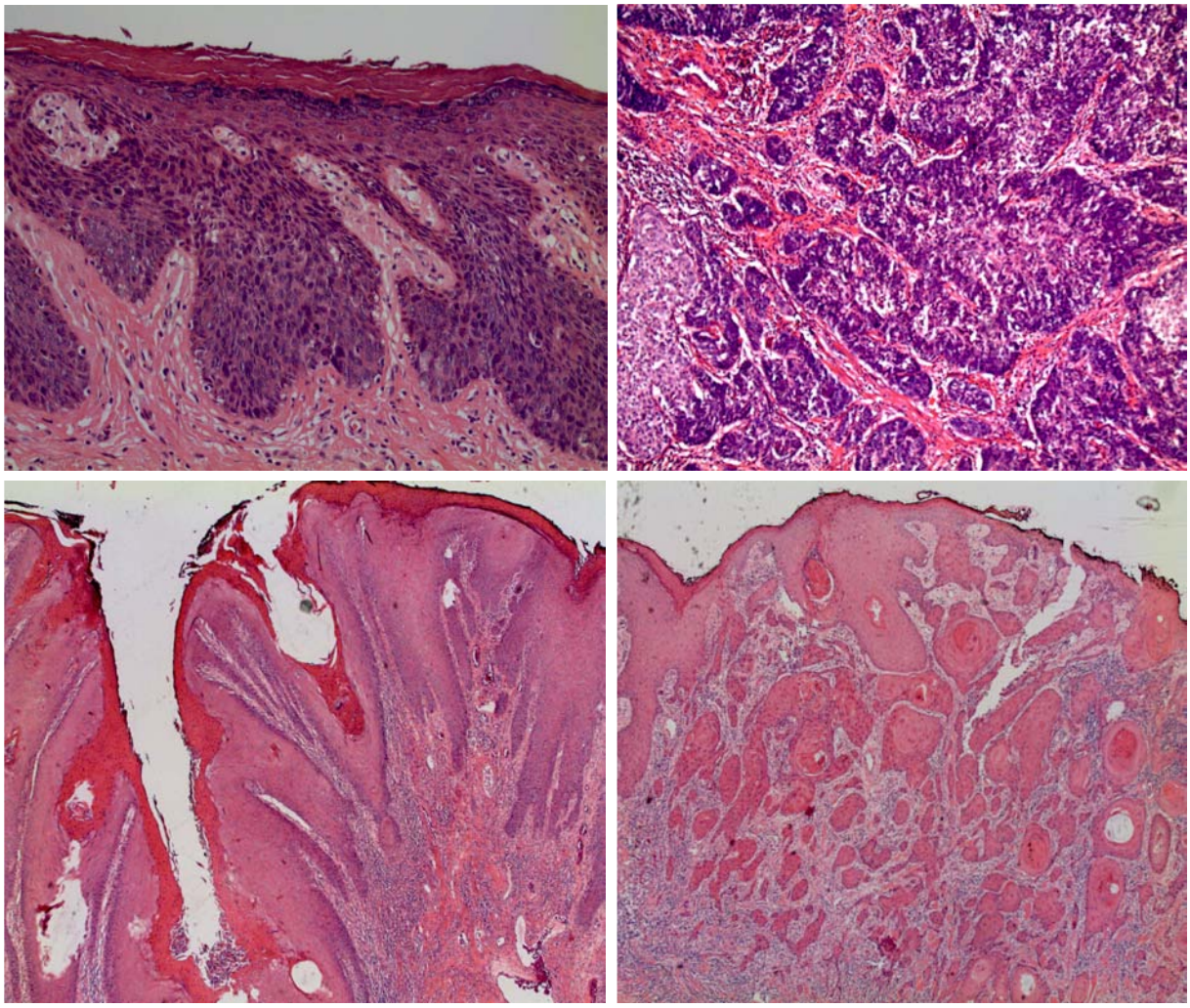


Figure 2 .*Histology of **HPV**-associated classical Vulvar Intraepithelial Neoplasia (VIN) III and Basaloid Vulvar Squamous Cell carcinoma (VSCC) in upper panel, and not HPV-associated differentiated VIN III and well differentiated keratinizing VSCC in lower panel. All specimens are stained with H&E.*

1.3 STAGING

The Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) has developed a system for staging gynaecological malignancies, including VC. The purpose of the classification system is to support clinicians in classifying and staging the tumours in order to plan treatment, provide an indication of prognosis, assist in the evaluation of the results of treatment and facilitate the exchange of information.

For VSCC, inguinofemoral nodal status is the most important prognostic factor, mainly dependent on the primary tumour size and depth of invasion [3, 33-45]. In 1988, the FIGO classification was changed from a clinical into a surgico-pathologic staging because of the inability to accurately predict lymph node status by physical examination (table 1) [46]. A revised FIGO classification system for VSCC was introduced in 2009 (table 2) [47, 48] consisting of four major changes [49].

Table 1. *The "old" FIGO classification of vulvar squamous cell carcinoma from 1988*

Carcinoma of the vulva	
Stage 1	Lesions ≤ 2cm in size confined to the vulva or perineum, no nodal metastasis
IA	Lesions ≤ 2 cm in size, confined to the vulva or perineum with stromal invasion≤ 1.0mm*, no nodal metastases
IB	Lesions ≤ 2 cm in size, confined to the vulva or perineum with stromal invasion > 1.0mm, no nodal metastases
Stage II	Tumour >2cm in largest dimension confined to the vulva or perineum, no nodal metastases
Stage III	Tumour of any size with adjacent spread to the lower urethra, the vagina, or the anus with unilateral regional lymph node metastasis
Stage IVA	Tumour invading any of the following: upper urethra, bladder mucosa, rectal mucosa, pelvic bone or bilateral regional nodes.
Stage IVB	Any distant metastases including pelvic lymph nodes

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

- Stage II (> 2cm) and IB (≤ 2cm) have been combined because these two categories of patients did not appear to differ in survival [39]. Tumours with negative node status are at low risk regardless the diameter of the tumour [35].
- Stage III represented a heterogeneous group of patients with both negative and positive lymph nodes. Because of their good prognosis patients with tumours involving the vagina and/or urethra with negative nodes [35, 38] are now classified as stage II (formerly stage III).
- Patients with positive nodes are still classified as stage III. The number of the involved nodes and morphology (size of the metastasis and presence of extra nodal growth is taken into account). An increasing number of positive lymph nodes and a larger diameter of nodal metastases both lead to a worse survival rate [34-36]. Nodal metastases with extra nodal spread have a worse prognosis compared to patients with the metastases confined to the lymph nodes [36, 37, 40] and are now classified as stage IIIC.
- The bilaterality of positive nodes has been discounted because it turned out not to be an independent prognostic factor when a correction is made for the number of positive lymph nodes [33].

In this thesis we have used the old staging from 1988 since the new staging was introduced in 2009 after the inclusion of patients was finished.

Table 2. *The" new" FIGO classification of vulvar squamous cell carcinoma from 2009*

Carcinoma of the vulva	
Stage 1	Tumour confined to the vulva or perineum,
IA	≤ 2cm in size with stromal invasion ≤ 1mm*, negative nodes
IB	≤ 2cm in size or with stromal invasion > 1mm, negative nodes
Stage II	Tumour of any size with adjacent spread (1/3 lower urethra, 1/3 lower vagina, anus), negative nodes
Stage III	Tumour of any size with or without extension to adjacent perineal structures (lower urethra, lower vagina, anus) with positive inguino-femoral lymph nodes
III A	Tumour of any size with positive inguino-femoral lymph nodes with 1 lymph node metastasis≥ 5 mm or 1-2 lymph node metastasis(es) < 5 mm
III B	With 2 or more lymph nodes metastases≥ 5 mm or 3 or more lymph nodes metastases < 5 mm
III C	With positive node(s) with extra capsular spread
Stage IV	Tumour invades other regional structures (2/3 upper urethra, 2/3 upper vagina), bladder mucosa, rectal mucosa, or distant metastases
IV A	Tumour invades any of the following: upper urethral and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to the pelvic bone, or fixed or ulcerated inguinofemoral lymph nodes
IV B	Any distant metastasis including pelvic lymph nodes.

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

1.4 SYMPTOMS AND DIAGNOSIS

Long-term pruritus is the most prominent symptom of vulvar cancer. Other common symptoms are lump or mass on the vulva, bleeding, pain, discharge or urinary tract symptoms [50, 51].

Clinical examination with palpation, inspection and histopathological examination of biopsies from all suspicious areas in the vulva such as warty, cauliflower-like lumps, ulcers and pigmented areas found the basis. Fine-needle aspiration of enlarged lymph nodes is done to verify metastases. MRI is used to explore involvement of the anal sphincter and adjacent tissues [52].

Today we have no non-invasive methods that can predict the presence of positive lymph nodes with accuracy. Not even ultrasound [53, 54], MRI, [52] CT or PET [55] can allow us to refrain from inguinofemoral lymph node resection [56-59] with exception for microinvasive tumours [60, 61]. The negative predictive value is not high enough.

The lymphadenectomy causes morbidity. The complication rate of complete inguinofemoral lymphadenectomy varies between 24 and 70% [62]. Common short-term complications are infection and wound breakdown while long-term complications include lymph oedema, lymphatic cysts and chronic cellulitis of the legs [63-66]. The lymphatic drainage of the vulva was described in 1983 [67], with a well defined direction of the lymphatic flow to the groins.

1.5 THE SENTINEL NODE CONCEPT

The sentinel node (SN) concept is based on the assumption that all spread from the tumour goes through one or several primary draining lymph nodes (SNs) [68-70]. If this/these are tumour free, all other nodes should be disease-free. Therefore, a non-metastatic SN may allow the removal of only this lymph node (figure 3). The SN approach is attractive since it could reduce complications caused by the lymphadenectomy but still allow staging of the tumour. SN procedure is an

alternative to complete inguinofemoral lymph adenectomy that reduces postoperative complications [71].

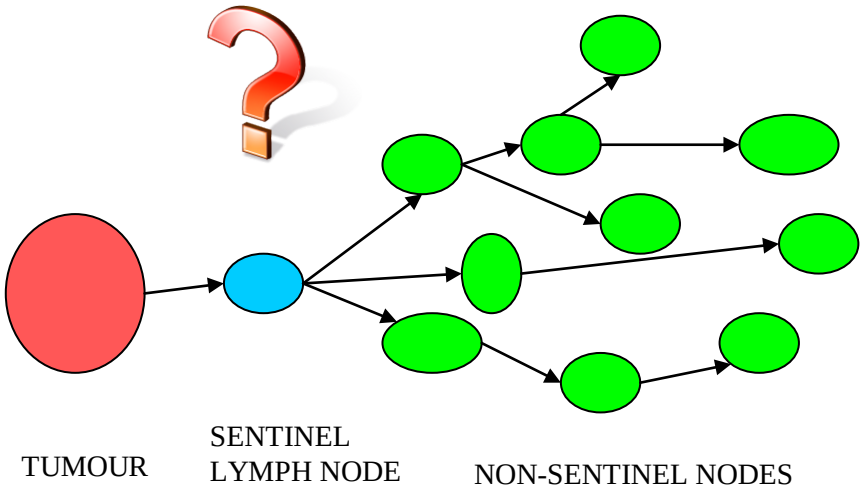


Figure 3. *The Sentinel Node (SN) concept. All spread from the tumour will pass the SN. If this node is tumour free all other nodes should be disease free, and can allow us to only remove the SN.*

In gynaecological surgery there are some experiences of the SN technique from cervical cancer, endometrial cancer and vaginal cancer [72-74], but its main use has been in vulvar surgery. Studies where the SN detection was followed by complete inguinofemoral lymphadenectomy suggest that the SN procedure is highly accurate in identifying lymph node metastases with a negative predictive value approaching 100% [68-70, 75]. The optimal design to prove safety and clinical utility would be an equivalent randomized trial (RCT) in which patients with a negative SN are randomly assigned to either observation alone or inguinofemoral lymphadenectomy. However, an RCT is difficult to perform due to low incidence. Recently, a large multicenter observational study provided level 3 evidence indicating that it appears safe to omit inguinofemoral lymphadenectomy in case of a negative SN [76]. Hopefully the follow-up study "Groningen International Study on Sentinel Nodes in vulvar cancer (GROINSS-V-II) trial" will answer the question of whether radiotherapy alone is sufficient treatment in cases with a positive SN.

In breast cancer the SN procedure is now routine [77]. In cutaneous melanoma SN is used for staging of the regional lymphatic nodes [78]. After an initial learning period the false negative rate in breast cancer is about 3 % [79], while the rate in vulvar cancer ranges from 0 to 6% [62, 68-70, 75, 80].

Although the presence of HPV has been examined in SN in cervical carcinoma and in lymph nodes in general [20, 23], we have not found any studies on the presence of HPV in SN in VSCC. The presence of HPV in SN has been suggested to be a diagnostic marker of metastatic involvement in cervical cancer [81, 82].

1.6 TREATMENT

1.6.1 Surgery

Surgery is the first choice of treatment; both in early and more advanced cases [83]. The treatment has gone from large "butterfly resections" of vulva and inguinofemoral lymph nodes en bloc to less radical surgery [84, 85]. In early cases wide local resection or vulvectomy and inguinofemoral lymphadenectomy through separate incisions has been the standard treatment [86, 87]. If the lesion is located $\geq$ 1 cm lateral of the midline, lymphadenectomy will be unilateral, otherwise it will be bilateral [41]. In stage IA (invasion depth <1mm) it is considered safe to omit inguinofemoral lymphadenectomy because of negligible risk of lymph node metastases [60, 61]. It is necessary to obtain at least 8mm pathological margins that coincide with approximately 2cm on fresh tissue [41, 85]. The aim is to get local control while sparing as much tissue as possible. Surgery is also performed in more advanced stages with lymph node metastases [88, 89]. Locally advanced disease comprises about one third of all cases at time of diagnosis [90]. Ultra radical surgery appears to be a reasonable treatment for locally advanced but resectable tumours, especially in the absence of nodal metastases. The surgical treatment is mutilating and causes sexual and psychological problems [91-93].

To reduce postoperative complications and mutilation the surgery has, during the past years been more individualised including radical local excision and preoperative SN technique [76, 94, 95].

1.6.2 Radiotherapy and Chemotherapy

Radiotherapy is given postoperatively if metastases in the regional lymph nodes and/or the tumour margins are not good enough. It can also be given as neoadjuvant therapy in combination with chemotherapy to shrink the tumour and make it operable; the possibilities of avoiding local organ resection increases and radiotherapy gives tumour control comparable to surgery in locally advanced disease [96-98].

Chemotherapy can be given alone as palliative treatment or combined with radiotherapy in a curative intention [99]. A combination of treatment modalities radiotherapy, surgery and chemotherapy can optimize the treatment [88, 95].

1.7 PROGNOSTIC FACTORS

1.7.1 Lymph node metastases

The presence or absence of lymph node metastasis in the groins represents the most powerful prognostic factor for recurrence and survival in VC [3, 33-45]. The new FIGO-staging from 2009 gives a much better estimate of prognosis for patients with VC [49].

Five-year survival is 70-90 % for women without metastases and 25-41% for patients with metastatic spread to regional lymph nodes [3, 33, 100]. The numbers of involved nodes further discriminate as well as periglandular growth [36, 37, 40]. Studies on histopathologic characteristics such as depth of invasion, tumour diameter, and localisation of tumour as well as lymph space involvement and grade have been performed with the aim to identify patients with low risk of lymph node metastases. The results have been contradictory [3] and it seems impossible to define a low risk group based on these factors. Only in patients with tumours with ≤ 1mm invasion is it accepted to omit inguinofemoral lymphadenectomy [60, 61].

1.7.2 HPV-status in tumours and SN

HPV-status seems also to influence the prognosis in VSCC [101]. A better prognosis for patients with HPV-positive tumours has been indicated by some studies [21, 23, 102-104], while other investigators found no prognostic significance associated with HPV status [20, 68, 105].

The prognostic significance of HPV DNA in lymph nodes in cervical cancer has been discussed and HPV presence has been suggested to be a diagnostic marker of metastatic involvement [81, 82].

1.7.3 Molecular markers

In order to improve the treatment and follow-up of vulvar cancer patients, the importance of various molecular markers has been studied [106]. However, so far no conclusion regarding the prognostic value of these markers could be drawn due to the

small numbers of patients included in each study, and lack of multivariate analysis. Moreover most studies have not controlled for HPV-status of the tumours.

1.7.4 Laminin 5 γ2 chain (Ln-5 γ2 - chain)

Tumour invasion is characterized by the tumour cells crossing the basement membrane (BM) marking the difference between a malignant and benign tumour [107]. Ln5 are molecules of the BM, which separates the epithelium from the underlying connective tissue. The laminin family is a group of large cross-shaped heterotrimeric proteins composed of three subunits; one heavy α-chains and two light β-chains [108]. At least 12 different laminins are identified [109]. They are synthesized by numerous cell types and expression of laminin isoforms are cell- and tissue specific [109] with different biological functions [108].
Ln-5 is produced only by the epithelial cells and consists of α3, β3 and γ2 chains. These three subunits are coded by specific genes: the LAMA3 gene on chromosome 18q11.2 (α 3) LAMB3 gene on chromosome lq32 (β 3) and LAMC2 on lq25-31 (γ 2) [110, 111]. Accumulating data suggest that ln5 is involved in tumour cell invasion and the isoforms (α3, β3 and γ2) plays an important role in epithelial cell adhesion to BM and in cell migration [108].

In normal tissues ln-5 γ2 chain has an important function in the static adhesion of epithelial cells to the BM through the attachments to integrins and the assembly of hemidesmosomes.
Studies have shown that the γ2 chain of ln5 is expressed by the migrating but not by the stationary keratinocytes in wound healing in skin [110]. In invading cancer cells over expressed ln γ2 chain appears to be present in a monomeric form as laminin α3 and β3 are absent or found in much lower levels.
Several immunohistochemistry (IHC) studies have reported that ln-5 γ2 chain expression is up-regulated in invading cancer cells, suggesting it as a marker of invasiveness in SCC and other epithelial carcinomas such as cervical, multifocal anogenital, and colon carcinoma [112-116]. Increased expression of ln5 γ2 chain has also been correlated with a more aggressive behaviour of the tumour [113, 117-121] and ln-5 γ2 chain has also been revealed as a marker for microinvasiveness in cervical and vulvar carcinomas [114, 115].

Based on this data the question arose if ln-5 γ2 chain expression could add prognostic information in VC.

1.7.5 DNA ploidy and proliferative markers

DNA ploidy is an established prognostic factor in endometrial and breast carcinomas [122, 123], and S Phase Fraction (SPF) and Ki-67 (MIB-1) in endometrial cancer [124, 125]. These parameters have been found to give additional information concerning prognosis and are widely used in decision making of treatment of these carcinomas. However the clinical implications of these factors are not yet clear in VC [115].

DNA ploidy

Two cytometric methods are generally used to determine ploidy - Flow Cytometry (FCM) and Image Cytometry (ICM). Both methods can be used on fresh frozen tissue or formalin-fixed paraffin embedded material. The most common method is FCM. The cell nuclei are stained with fluorochrome and quantification of the nuclear fluorescence is done by the flow cytometer. The amount of DNA in the cell is assumed to be equal with the amount of fluorescence [126]. An advantage with this method is the possibility of rapid analyses of large cell populations (20000 nuclei per specimen). A disadvantage with FCM is the inability to separate normal and tumour cells.

ICM is based on Feulgen-staining, a method first described by Feulgen and Rosenbeck 1924 [126]. The DNA staining is measured on microscopic slides and 100-500 whole cells are selected for analysis. By this method it is possible to analyse small cell populations with large stromal components which can be difficult with FCM since they can be overlooked.

DNA ploidy has been used in predicting tumour behaviour and prognosis in various malignancies [127-129]. Quantitative measurements of DNA content are used in order to detect major abnormalities in DNA content (DNA ploidy). The normal human somatic cell with 46 chromosomes (23 pairs) is referred to as diploid. A cell with fewer or more than 46 chromosomes are referred to as aneuploid. In general, peridiploid (near diploid) tumours, i.e. tumours with only minor deviations in DNA ploidy, compared with normal diploid tissue, have a good prognosis in many

malignancies, whereas major aneuploid tumours are associated with poor survival [127, 129].

S- Phase Fraction (SPF)

Although there is no single optimal method of measuring the proliferative activity in tumour cells, estimation of the SPF by FCM has been of prognostic importance and widely used [130]. S phase is a part of the cell cycle in which DNA synthesis occurs before mitosis. Measuring the SPF by FCM determines the percentage of cells in this phase of the cell cycle [131, 132] and gives a measure of the proliferation.

Ki-67

The proliferative capacity of tumour cells is the fundamental feature of growing tumours, and estimates of cell proliferation provide more objective prognostic information. The growth of a tumour is determined by the balance between proliferation as the growth fraction and cell cycle time on one hand, and cell loss, which is the result of apoptosis and necrosis on the other.

The Ki-67 gene encodes for the nuclear Ki-67 antigen which is present in all phases of the cell cycle except G_0 and the early part of G_1 [133, 134]. Thus the Ki-67 antigen is present in proliferating cells and absent in resting cells. IHC methods for detection of antigens associated with cell proliferation have been developed. A monoclonal antibody against the Ki-67 antigen (MIB1) has proven valuable by allowing direct monitoring of the growth fraction of normal and neoplastic cells in routinely formalin-fixed paraffin-embedded specimens by IHC detection [135].

1.8 PROTEOMICS

After the human genome sequence was determined, the interest moved to the analysis of the protein complement of the genome (proteomics) [136]. The term proteome was first coined by Wilkins et al 1994 [137] and refers to all proteins in a cell, tissue or organism. The correlation between DNA sequence and proteins is low, reflecting alternative splicing as well as post-translational modifications, which explains the existence of a huge number of proteins. Simplified it can be said that the genome describes what can happen, the transcriptome what appears to happen, the proteome what makes it happen and finally the metabolome what has happened and is happening giving the phenotype [138].

As in many other cancer forms, biomarkers for diagnostics prognostic and therapeutic guidance are needed in VC. During the last years, different genes have been traced that, in changed conditions can not only induce tumours but also affect their spread and growth. It is difficult and sometimes impossible to connect specific cell functions to specific gene alterations since each gene can produce several kinds of proteins.

1.8.1 Two dimensional gel electrophoresis (2 DE)

Using 2 DE, proteins are separated in two dimensions based om the isoelectric point p*I* and size. In the first dimension a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pHs other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. In the second dimension is the relative molecular mass determined using sodium sulphate-polyacrylamide gel. The proteins are visualized in the gel by staining with e.g. Brilliant blue or silver. Protein spots of interest are identified using mass spectrometry [139, 140].

1.8.2 Mass spectrometry (MS)

Recently MS based workflow has become increasingly common and is used to study protein identification and quantification.

Briefly, a mass spectrometer separates ions in the gas phase based on their mass to charge ratio (m/z). All mass spectrometers are essentially composed of three major components; an ion source, a mass analyzer and a detector (figure 4).

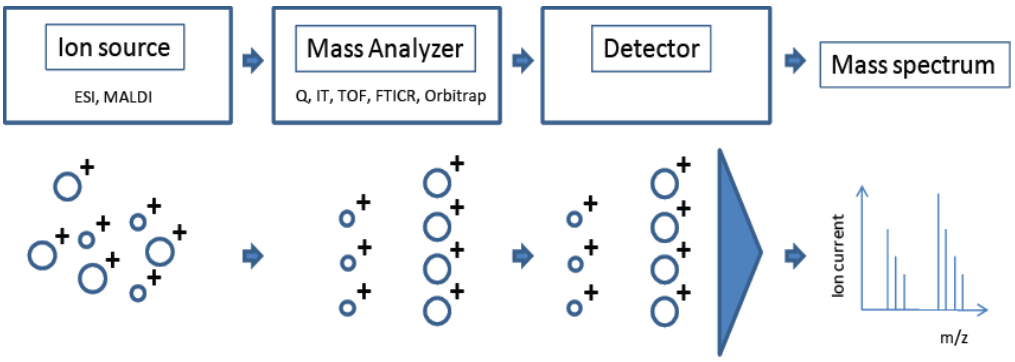


Figure 4. Schematic overview of a mass spectrometer (reproduced with permission from the thesis of Maria Pernemalm)

There are two different approaches in MS based proteomics. In top-down proteomics, intact protein ions or large protein fragments are subjected to gas-phase fragmentation for MS analysis. In bottom-up approaches, purified proteins, or complex protein mixtures are subjected to proteolytic cleavage, and the peptide products are analyzed by MS.

To help interpret the proteomics data, which comes out as a long list of identified and quantified proteins, bioinformatic tools have to be used. Example of such databases are Gene ontology (GO) and The Database for Annotation, Visualization and Integrated Discovery (DAVID) [141]. In comparative proteomic studies, the data mining steps are often confined to proteins measured in all samples as protein quantification generally is made relative to the other samples. Comparative clinical proteomics has to deal with large inter-individual variation. Consequently patient groups often need to be large in order to detect differences between groups. Although many proteins are detected in each sample, protein overlap is not necessarily large.

2 AIMS OF THE STUDY

The general aims of this thesis were to get increased knowledge of etiologic, diagnostic and prognostic factors in vulvar cancer.

The specific aims of the individual studies were:

- To assess the clinical value of a preoperative lymphoscintigram during the SN-procedure, and to determine the detectability of SN and the false negative rate using radio colloid and blue dye.

- To evaluate the proportion of HPV-positive and HPV-negative VSCC including SN, and to compare survival in the two groups.

- To investigate the prognostic impact of ln-5 γ 2 chain expression in relation to clinicopathological factors and HPV status as well as DNA ploidy and proliferative activity (Ki-67, SPF) in primary VC.

- To explore the proteome characteristics of HPV- positive versus HPV-negative VC, protein and pathway profiling on a global and individual tumour level searching for markers reflecting recurrence.

3 MATERIAL AND METHODS

3.1 STUDY SUBJECTS

All four studies are based on a group of 85 women with invasive VC, T1-T3 without palpable lymph nodes who were operated at Karolinska University Hospital, Solna by (two surgeons) during the years 2000 to 2007 (table 3).

Paper I and II
The 77 women who underwent surgery with the SN procedure
Paper III
The group of 85 women who underwent surgery due to vulvar cancer
Paper IV
Fresh frozen tissue samples from a subset of 7 HPV -positive and 7 matched HPV-negative vulvar tumours.

Table 3. Characteristics of the 85 participating women with invasive VC, T1-T3

Variable	**number**	**%**
Age (years) Mean	71,4	range 40-92
FIGO stage		
I	20	23.5 %
II	36	42.4 %
III	23	27.1 %
IV	6	7.0 %
Position of the tumour		
Lateral	30	35.3%
Central □	55	64.7%
Tumour length (mm)	28.06	range 1-95
Tumour width (mm)	22.12	range 1-70
Operation in vulva		
Vulvectomy	38	44.7%
Hemivulvectomy	29	34.1%
Wide local excision	18	21.2%
Operation in groins		
Unilateral	25	30.6 %
Bilateral	54	63.5 %
Histopathology		
well differentiated	18	21.4 %
moderately differentiated	28	33.3 %
poorly differentiated	37	44.7 %
unclassified	1	1.2 %
Infiltration deep mean mm	9.5	range 1-45
LVSI^{o}	20	23.5%
Lymph node metastasis	24	28.2%
Periglandular growth	9	10.6%
Surgical margins		
Intact	75	88.2%
Invaded	10	11.8%
HPV-status		
HPV-pos	29	65.5
HPV-neg	55	33.3
missing	1	1.2

□ defined ac < 1cm from midline. ^{o} Lymph Vascular Space Involvement.

3.2 SENTINEL NODE IDENTIFICATION AND SURGICAL TREATMENT

Patients underwent preoperative lymphoscintigraphy and injection of blue dye, alternatively only blue dye. Lymphoscintigraphy was performed in the morning of the day of surgery, 40 mBq human serum albumin colloid, labelled with ^{99m}Tc (NANOCOLL, Nycomed Amersham Sorin S.r.l., Saluggia (Vercelli) Italy), was injected intradermally at four sites around the tumour. Immediately thereafter, a 30-min dynamic A-P acquisition was initiated with 1-min frames using one head of a Triad XLT gamma camera equipped with a parallel-hole collimator (Trionix Inc., Twinsburg, OH, USA). This was immediately followed by a static 5-min acquisition. The site(s) of the detected hot nodes were marked on the skin and the patients were operated 2-5 hours after the injection. Fifteen minutes prior to the skin incision, blue dye (metyltionincloride, 10mg/ml, ATL, Sweden) was injected intradermally around the tumour at the same four sites as used for the injection of the radioactive tracer. The groins were explored using a handheld γ-probe (Europrobe®, Eurorad, France) and dyed lymph nodes were identified macroscopically. Each lymph node containing radioactivity and/or blue dye was removed and considered as an SN. Thereafter, a complete inguinofemoral lymphadenectomy was carried out; bilateral excision was performed if the tumour was central (<1 cm from the midline), otherwise the excision was unilateral. Finally a vulvectomy or wide radical excision was performed. All patients with positive lymph nodes received subsequent adjuvant treatment with radiotherapy.

3.3 HISTOPATHOLOGICAL EXAMINATION

All SN specimens were sent separately for histopathological examination. They were cut in 2-3mm slices, or if larger than 4mm, divided in two along the axis, and then paraffin embedded. All blocks were serially step-sectioned at levels: one section per 500 µm, 4 µm thick, three sections at each level were stained with H&E. If no cancer was detected, the last one was used for IHC with cytokeratin antibodies to exclude metastases. We used standard sectioning and H&E staining of the primary tumours and the non-sentinel nodes.

3.4 PCR FOR DETECTION OF HPV DNA

DNA was extracted from 30µm pre-treatment paraffin embedded tumour slices with the High Pure RNA paraffin kit (Roche Diagnostics), [142]. Blank samples were

taken and treated in the same manner to rule out cross-contamination between samples. Amplifiable DNA was confirmed by PCR for the human housekeeping gene [142]. Presence of HPV DNA was tested by using general primer pairs, GP5+/6+ and CPI/IIG, as described [143, 144]. In addition, all samples were tested by HPV -6, -11, -16, -18 and -33 type specific primers [145] to detect false HPV-negative samples by general primers. Samples positive for HPV with general primers and negative for HPV type specific primers, were sequenced for determination of the HPV type on the amplicon generated from these primers, as described by Mellin et al.[146].

3.5 IMMUNOHISTOCHEMISTRY (IHC)

IHC studies of Laminin-5γ2-chain and Ki-67 as well as the analyses of STAT 1, PSMA 5, MX 1, and LGMN –proteins found by proteomics was performed using the standard peroxidase avidin-biotin-complex technique (Vector, Elite Standard Kit, catalog PK-6100, Vector Laboratories, Inc., Burlingame, CA) described earlier [115, 129, 147].

Sections of 4 µm thick paraffin-embedded specimens of the tumours were examined.

We used monoclonal antibody (Dako, Mo a Hu laminin- 5 γ2 chain, Clone 4G1) for Ln-5γ2-chain**,** monoclonal mouse anti-human Ki-67 antigen, clone MIB-1 for Ki-67. Primary antibodies against STAT1, PSMA5, MX1 and LGMN were generated by the Human Protein Atlas Project [148] .

The IHC reactions was estimated and visually scored (0 to 3+) by two investigators from whom the histopathological data of the patients was unknown. To declare a lesion positive, >1% of the cells had to show distinct cytoplasmatic immunoreaction and be regarded as positive.

The evaluation of ln-5γ2-chain, Ki-67 was estimated and categorized as described previously [115, 147] and as follows: negative ($\leq$ 1% of the tumour cells were positive) + (low) = (> 1% to $\leq$ 25% of the tumour cells were positive); ++ (moderate) = (> 25, 1% to $\leq$ 75% of the tumour cells were positive); +++ (high) = > 75% of the tumour cells were stained. The evaluation of STAT1, PSMA5, MX1 and LGMN was given in percentage of the stained tumour cells per total area of the tumour cells of the sample preparation. Staining intensity was grading using a scale from 0-3; with 0 representing negative staining and 3+ strong staining.

3.6 DNA PLOIDY AND S-PHASE FRACTION BY FLOW CYTOMETRY

A previously published improved Hedley-method [149]) for the preparation of paraffin-embedded tissues for flow cytometric DNA analysis has proved useful for the examination of various types of tumour tissues [150].

DNA ploidy analysis and SPF was performed in paraffin-embedded formalin-fixed tumour specimens from 85 women with VSCC, and 67 were evaluated. Histopathological Sections (80 µm) were cut, and dewaxing and rehydrating were done by running the machine overnight. Two changes of xylene and two changes of ethanol in a sequence of 100, 95, 70, and 50% were made at room temperature. Each incubation lasted 1 hour.

The Sections were then washed twice in distilled water for 20 min and were finally placed into 1 ml subtilisin Carlsberg solution for enzymatic digestion (0.1% Sigma protease XXIV, 0.1 M Tris, 0.07 M NaC1, pH 7.2) in a water bath at 37°C. The incubation time was 0.5 to 2 hours depending on the type of tissue. The yield of cell nuclei in the solution was checked microscopically. After incubation without shaking, the samples were finally shaken for 20 min.

The samples were analyzed after a staining time of at least 1 hour and up to 8 hours at room temperature. Staining was carried out by adding 1 ml DAPI-phosphate solution (10 µM DAPI, *800* mM disodium-hydrogenphosphate) directly to the 1 ml subtilisin Carlsberg solution containing the sectins and the suspended nuclei (final concentrations in the mixture: ***5***µM DAPI, 0.05% subtilisin Carlsberg, 400 mM disodiumhydrogenphosphate, 0.05 M Tris, 0.045 M sodium chloride, pH 8.2).

The samples were analyzed with a LSRII Flow cytometer (BD Bioscience, Stockholm, Sweden). The fluorochrome DAPI was excited in the ultraviolet (350-400 nm) and the fluorescence was measured in the blue region (>435 nm).

Usually at least 10,000 nuclei were analyzed from each sample at a measuring rate of about 500 nuclei/sec. If, for example, diploid nuclei are measured in channel number 70, a broad range of higher ploidies can then be analyzed in the same histogram. The corresponding G1 and G2 peaks can be easily correlated to each other using the linear x-scale.

A multicycle program applied for histogram analysis (Phoenix Flow Systems, San Diego, CA, USA) was used for calculating the percentage of nuclei after subtraction of sliced nuclei background.

3.7 PROTEOMIC ANALYSES

3.7.1 Protein digesting and iTRAQ labelling

Stable isotopic labels, iTRAQ (isobaric tags for relative and absolute quantification) were used for mass spectrometry based quantification. Each tumour sample was digested with trypsin and labelled with 8-plex iTRAQ. Pre-fractionation by isoelectric focusing to increase proteome coverage.

To reduce sample complexity, the iTRAQ labelled peptides were separated based on their isoelectric point, and only peptides in the p*I* interval 3.7-4.9 were used for identification and quantification.

3.7.2 Liquid chromatography – Mass Spectrometry/Mass Spectrometry (LC-MS/MS) analysis

LC-MS/MS is bottom-up proteomics preceded by LC. An online HPLC-MS performed on a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). A reversed-phase separation was done. The peptides were then analyzed by tandem mass spectrometry MS/MS in the Orbitrap where the peptide sequences were determined. The peptides were separated according to mass partially fragmented into amino acids and the fragment spectra together with the precursor mass is then used to determine the amino acid sequence of each peptide.

3.7.3 Protein identification and quantification

All MS/MS spectra with the identified peptides were searched against protein sequence databases to match the peptide sequences with known protein sequences, Mascot 2.2 [151] (Matrix Science Limited, London, UK) under the software platform Proteome Discoverer 1.1 (Thermo) against the NCBInr human target decoy (20090118). As decoy the reversed sequences were used. Searches were also done against a merged human and virus target decoy database. There were however no virus hits, so subsequent searches were done against a database composed of only human sequences.

Quantification of iTRAQ-8plex reporter ions was done by Proteome Discoverer (version 1.2) on the tandem mass spectra.

The scores and the original peptide lists were then further curated using the in-house developed software PQPQ . The program uses the quantitative pattern of samples to match peptides to proteins, based on the assumption that peptides originating from the same protein show a similar pattern. This makes it possible to use also low-scoring

peptides and low- intensity peptides without compromising in confidence. All protein quantified by PQPQ had an identification based on at least two peptides, and at least one of the peptides being above the score limit.

3.8 STATISTICAL AND DATA ANALYSIS

Paper I and II

Statistical analyses were performed using SPSS 17.0 for Windows (Chicago, Ill.USA 2008).

Means between groups were compared using Student's independent t-test and distribution between groups using the chi-square exact test. Survival was evaluated using the life table method taking censored observations into account.

Univariate comparisons were made using the Gehan-Wilcoxon method.

Multivariate survival analyses were performed using Cox regression.

Weighted kappa was calculated to give a measure of agreement between SNs found at scintigraphy and those found at surgery [152].

Paper III

Statistical analyses were performed using SPSS 18.0 for Windows (Chicago, Ill. USA 2008).

Disease-specific survival was evaluated with Cox regression model.

Relations between considered factors were analysed with Spearman`s correlation. Survival graph was constructed using the Kaplan-Meier method and tested with Gehan-Wilcoxon exact test.

Paper IV

Students t-test was performed using SAM (Significance Analysis of Microarrays) for Excel version 3.09 [153].

Multivariate statistics and modelling [154] was performed with SIMCA (SIMCA-P+ 12.0, Umetrics, Sweden).

The unsupervised principal component analysis (PCA) [155, 156] was done to obtain an overview of the data, detect clustering of the data and to pick up outliers if any.

The supervised analysis (orthogonal partial least square analysis, O-PLS) [157] of the dataset was used for classification and identification of proteins separating HPV-positive from HPV-negative or relapse from relapse-free tumours/patients.

Hierarchal clustering 2-ways; protein ID and sample was performed using the freeware Genesis version 1.7.6 [158]. This is a statistical method that is based on measured variables capable of identifying relatively similar groups of samples.

Gene Ontology (GO) annotation was performed using ProteinCenter™ (Proxeon Biosystems, Odense, Denmark) and The Database for Annotation, Visualization and Integrated Discovery (DAVID) [141] .

Pathway analysis to further examine protein quantity alterations, network building as well as matching to canonical pathways was performed using the web based software from Ingenuity Systems; Mountain View, CA, USA (Ingenuity Pathway Analysis, IPA, www.ingenuity.com). In brief, matching of proteins from the data set was done against the Ingenuity pathway database of known (canonical) pathways. The degree of matching was ranked by p-values. The analysis was performed both on a data subset consisting of only proteins shared between all samples, as well as on protein profiles from individual tumours (figure 6).

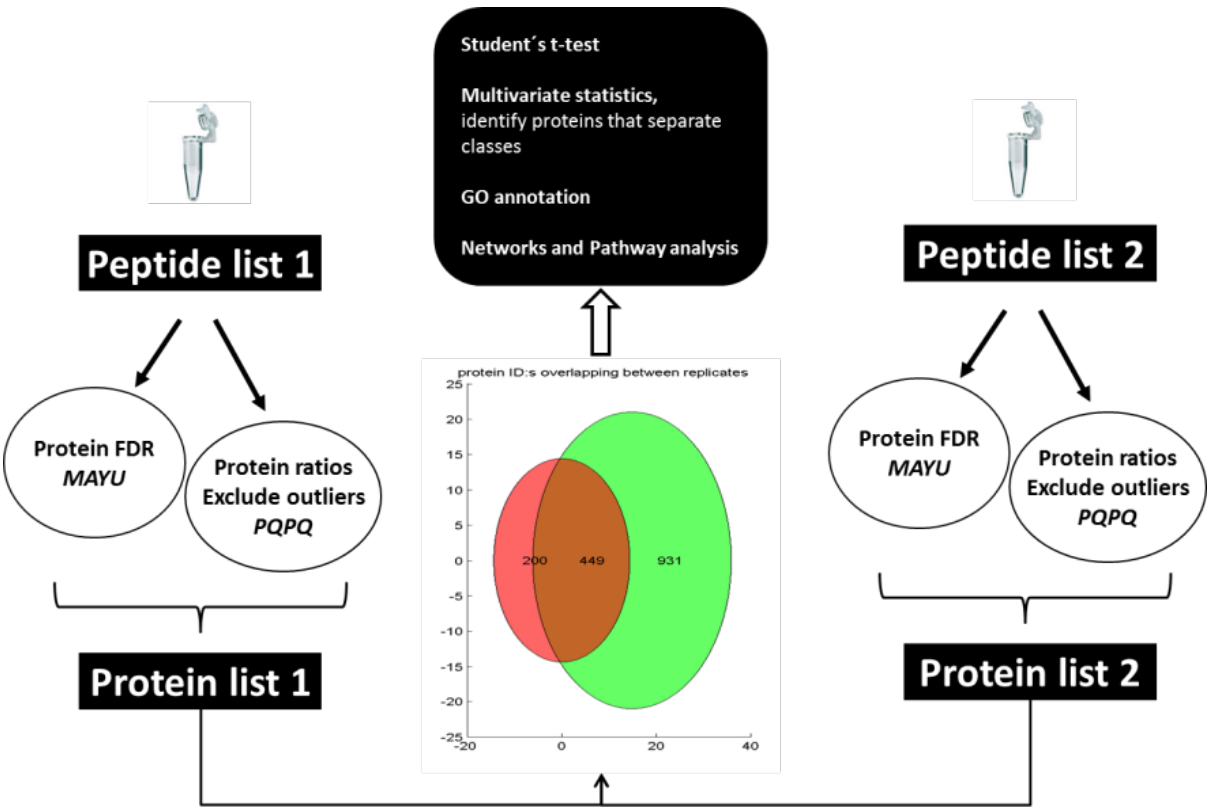


Figure 6. An overview of the data analysis workflow in the proteomic analyses

4 RESULTS AND DISCUSSION

4.1 PREOPERATIVE LYMPHOSCINTIGRAPHY AND SENTINEL NODE

A total number of 130 groins in 77 patients were operated. Our study is one of the largest performed at one single centre (n=77) [68-70, 75, 159]. All patients were operated by one or both of two surgeons. With the intention of obtaining experience and since a complete lymph node dissection was always performed, we allowed wide inclusion criteria. Today, we have learnt from our own experience as well as from others' that some patients are not suitable for the SN technique [76].

4.1.1 Preoperative lymphoscintigraphy

In two cases SNs were not visualized by lymphoscintigraphy presurgery, however, in one of these, SNs were identified with blue dye during surgery. In one of the 17 cases where blue dye alone was used, no dyed node was found. Thus the detection rate for the combined method with both radioisotope and blue dye was in our material 98% compared to 94% with blue dye alone that is similar to other reports [69, 160, 161].

The relation between the number of SNs shown on the scintigram and the number of SNs identified at surgery showed good agreement using weighted kappa [152].

Preoperative scintigraphy is in our experience helpful to identify the location and numbers of the lymph nodes especially when several SNs are visualized. The preoperative scintigram gives the best estimate available for detection of the accurate number of SNs. In some cases, however, we were not able to detect all the nodes that were shown on the scintigram and in some cases we found a larger number of SNs during operation than had been shown on the scintigram. One possible explanation could be that the nodes are located at different depths and thus hiding each other on the scintigram. In the ALMANAC trial (breast cancer) they found that SN biopsy-success decreases when there is non-visualisation of hot nodes on the pre-operative lymphoscintigram. [162, 163]

4.1.2 Identification of SN

During surgery at least one SN was identified in 94 of 126 groins in (in 75 patients excluding two patients with unsuccessful detection). Twenty-three patients (31%) had metastases in the groins and out of them 7 were bilateral, giving 30 affected groins. The SNs contained metastases in 20 groins. Two of these were micro metastases found by IHC. In 13 groins the SNs were the only positive node(s).

4.1.3 False negative SN

Out of 74 groins with a negative SN, two contained positive non sentinel nodes. This is in agreement of other results, thus false negative rate was 2.7% [62, 68-70, 75, 80]. In breast cancer surgery the rate of false negative SN decreases from 10% to 3% if the first 30 operations of each surgeon are excluded [79]. Our two false negative SNs appeared in 2002 and 2005, respectively. A possible explanation for false negative SNs is that the SNs are full of cancer and the nanocolloid pass through the SN to other lymph nodes [76]. The two false negative cases both had large midline tumours and one of them was multifocal. Both patients had 3 and 5 metastases respectively in the groin with negative SNs a phenomenon reported earlier [164]. Furthermore multifocal lesions have shown to be less suitable for the SN method [76]

4.1.4 Direction of lymphatic drainage

Lymphatic drainage was unilateral in 40 out of 58 patients (69 %) who had a successful lymphoscintigraphy, including all 16 patients with lateral lesions. Out of 42 patients with midline tumours, only 18 (43%) had bilateral lymphatic drainage at scintigram. This is in agreement with other studies [68, 75, 165, 166]. We did not differ between tumours close to midline and tumours abutting the midline as they did in a study by Haupsy [75]. In clinical practice (before sentinel node) the general rule has been to perform bilateral ingunofemoral lymphadenectomy in women with tumours <1cm from midline. The results of van der Zee et al and Haupsy et al indicate that only tumours abutting the midline have bilateral lymph flow, and it is safe to refrain groin dissection in the contra lateral groin without detectable SN in cases with tumours close to midline (<1cm) but not engaging midline [75, 76].

4.1.5 Size of tumours

Large tumours are more likely to have metastases which can change the lymphatic drainage [164, 167]. The only patient with a small (15mm) primary tumour on the left area of clitoris had previously undergone a local excision and the remaining tumour was 6mm. It has been reported that a scar after a previously removed lesion can hamper the SN identification in the groins [69] while this is not the experience of others [75].

In a European multi centre observational study by van der Zee et al, 259 patients with unifocal vulvar tumour ≤4cm and negative SN were followed up for 2 to 87 months with respect to groin recurrence. Only six recurrences (2.3%) were diagnosed [76]. If we have used same selection criteria as in this study only one of our patients with false negative or unidentifiable SN would have been included.

4.2 DISTRIBUTION OF HPV

HPV DNA was detected in 23 (31%) of the primary tumours. Patients with HPV-positive tumours were significantly younger (mean age 64 years) than those with HPV-negative tumours (mean age 75 years) and they also had smaller lesions. Single infections with HPV 16, 18, 33 and 52 were detected in 16, 2, 2, and 1 of the tumours respectively. In addition, there were two double infections, one with HPV 16+33 and the other with HPV 18+33. Ten (43%) of the 23 patients with HPV-positive tumours also had HPV in their SN.

Seven out of ten HPV-positive SN from HPV-positive tumours contained HPV 16, and one typed HPV 18, in concordance with the types in the tumours. In the two remaining cases it was impossible to classify the HPV type by sequencing. In addition, one patient with an HPV-negative tumour had an HPV-positive SN of type 16.

To our knowledge, this is the first report on HPV prevalence in VSCC in Sweden. Data from other Nordic countries shows similar results. In a report from Finland a prevalence of 36% of HPV in VSCC was found [18], while a report from Norway [21], showed 22% HPV prevalence. Furthermore, a report from Denmark demonstrated a prevalence of 52% in a subset of 60 patients diagnosed 1993-1998 [22]. This previously published data is fairly similar to ours but there may be some sources of error when comparing HPV prevalence data, such as the selection of VSCC patients according to age or survival. In the Danish study there may have been considerable selection since only surviving patients were included, which is reflected in the low mean age (63 years) of the patients. This may also explain the high prevalence of HPV. Another confounder may be the fact that the materials compared were collected during different decades. A recent report on tonsillar cancer in Stockholm, Sweden, showed that there has been a significant increase in HPV prevalence during the last decades, and even between 2000 and 2007 [102, 103]. It may be possible that the same trend is also true for VSCC. This may explain the

slight discrepancy between our data and the Norwegian data collected between 1977 and 1991 [18, 22]. An additional confounder may be different detection methods and patient numbers. DNA in formalin-fixed, paraffin embedded tissue specimens is often degraded to fragments smaller than 250 base pairs because of fixative-induced cross-links. Only PCR assays generating relatively short PCR products can be reliably applied to this material.

Various PCR and read-out assay combinations display variable analytical properties, particularly in terms of sensitivity for the detection of the HPV types. The method we used works with 188 base pair. Our material is relatively fresh and collected during less than a decade. In concordance with previous studies, HPV 16 was the dominant type in the tumours and SN, and no tumours were positive for low-risk HPV types [23, 105] .

4.3 SIGNIFICANCE OF HPV FOR SURVIVAL AND METASTASIS

We found that patients with HPV-positive primary tumours had significantly better survival than those with HPV-negative tumours (p=0.001). Even when adjusted for age and tumour size the p-value was 0.030. Disease-specific survival was significantly different with respect to HPV status (p=0.004, exact test) (Figure 7). In fact none of the patients with an HPV-positive primary tumour died of disease, while 18/52 patients with HPV-negative tumours died during the follow up period, in spite of the lack of difference between the proportions of lymph node metastases between the two groups (35% and 26% respectively). A better prognosis for patients with HPV-positive tumours has been indicated in some studies [21, 102, 103], while other investigators found that HPV status had no prognostic significance [20, 68, 105]. Recurrence-free survival in relation to HPV did not reach significance when age and tumour size was included in the statistic analysis.

In patients with HPV-positive tumours, metastatic SNs were significantly more often HPV-positive (5/5) as compared to those without metastases (5/18) (p=0.007) (Figure 7). No prognostic significance of HPV presence in SNs was detected; however the size of the material is too small to draw any conclusions.

Notably, we also observed that HPV was present in one case in an SN, but not in the corresponding primary tumour. This was confirmed for both the SN and the primary tumour by Multiplex Luminex [168]. The HPV-positive SN in the patient with an

HPV-negative tumour might be explained by an HPV infection not correlated with the malignancy.

To our knowledge, the presence of HPV in SN has never before been investigated in VSCC, although previous studies have investigated the presence of HPV DNA in lymph nodes in general [20, 23]. Pinto and co-workers assessed HPV status retrospectively in lymph nodes in a subset of 30 cases of HPV-positive and 13 cases of HPV-negative VSCC, and found HPV in lymph nodes from 10 patients with HPV-positive tumours. In all 10 cases, lymph node metastases were also found [20]. Sutton *et al*. tested metastatic nodes from 11 cases of HPV-positive tumours and detected HPV in 9 of them [23]. This is in accordance with our results that 5/5 metastatic SNs contained HPV (figure 8).

In cervical cancer, HPV status in SNs has been examined by Coutant and co-workers in 59 patients [81]. They found that HPV DNA was more frequently found in metastatic SNs compared to non-metastatic SNs. Lee and co-workers [82] examined 57 patients and found that absence of HPV in SN had negative predictive value for lymph node metastasis and recurrence.

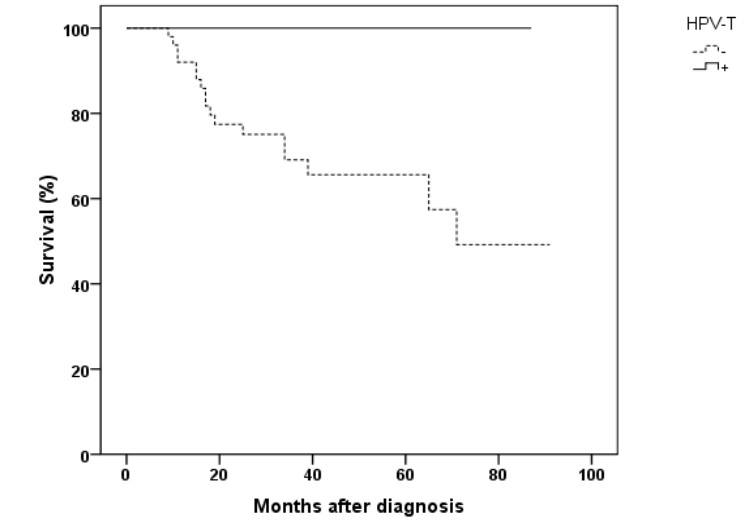


Figure 7. Disease specific survival by HPV-status of the primary tumour (p=0.04, exact test)

Thus, data from HPV testing of lymph nodes in general in vulvar cancer as well as the testing of SNs in cervical cancer are in agreement with our results. HPV positivity in lymph nodes in vulvar and cervical cancer is associated with metastatic involvement. The prognostic significance HPV DNA in pathologically negative lymph nodes (LN) is uncertain. It can be speculated that they represent a clinically significant group of micro metastases that have not been detected or caused by migrating disintegrating tumour cells that contain viral DNA. In cervical cancer a large prospective study has confirmed a direct correlation between the presence of HPV in LN and worse prognosis [169], whereas other small retrospective studies did not [170]. Maybe HPV typing of SN could play a supportive role to reduce the false negative rate in patients with HPV-positive tumours. Larger studies with longer follow up and standardization of testing native and formalin embedded material are needed to evaluate the prognostic significance of HPV DNA in SN.

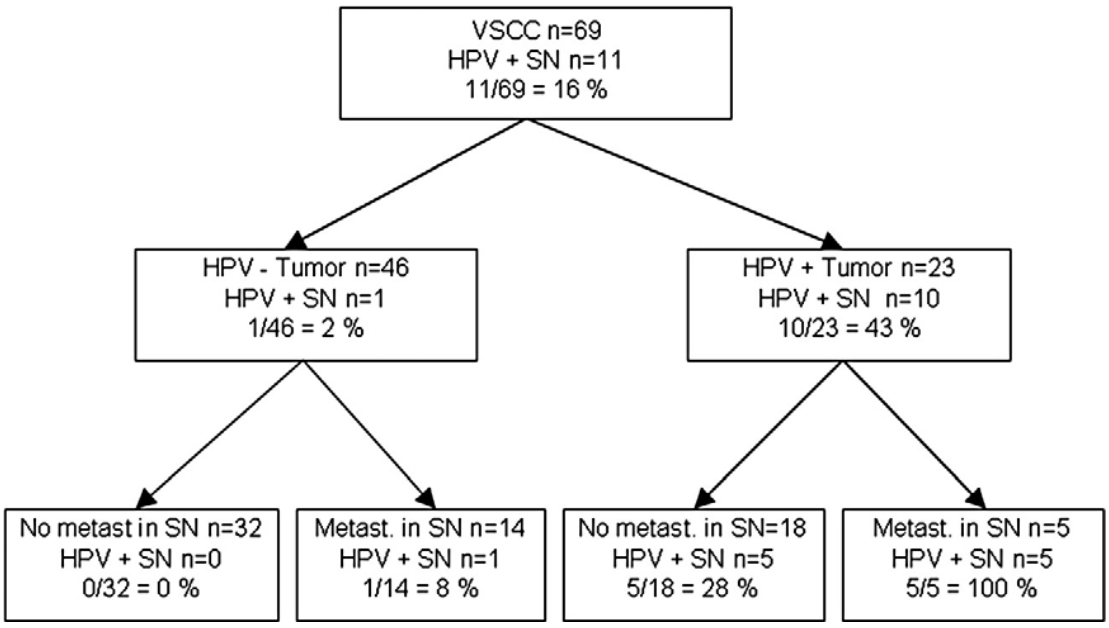


Figure 8. HPV-positivity (%) in SN of 69 VSCC

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4.4 SIGNIFICANCE OF MOLECULAR MARKERS AND PLOIDY

4.4.1 Laminin-5 γ2-chain

The staining was observed in cytoplasm of the cancer cells along the invasive front and to isolated cancer cell clusters invading the stroma. Ln-5γ2-chain expression was not found in the stromal cells or in the basement membrane.

Out of 76 tumours evaluated for ln-5y2-chain, seven (9.2%) tumours showed no expression, while 30 (39.5%) tumours showed low, 20 (26.3%) moderate and 19 (25.%) high expression respectively. In the statistical analyses in survival tumours with ln-5y2-chain no, low or moderate expression were grouped together and compared to tumours with high ln-5y2-chain expression.

Ln-5y 2 chain showed positively significant correlation with stage (p=0.044), tumour-size (p=0.012), differentiation grade (p=0.003), metastases (p=0.016) and was negatively associated with HPV status (p=0.034). High expression of ln-5y 2 chain was more often seen in HPV-negative tumours than in HPV-positive tumours.

In the univariate analyses regarding the different factors, ln-5y2 chain and HPV-status had significant impact on survival. Analysing these two factors together in a Cox regression model, both of them showed independent impact on survival.

When considering other clinical and histopathological factors showing impact on survival and including them in a stepwise logistic Cox regression analysis only HPV status and tumour stage were significant factors for survival. Competing non significant factors were age, tumour-size, metastases, and ln-5γ2 chain.

Various polyclonal and monoclonal antibodies against the ln5 γ2 chain have been used as markers of invasive cancer in e.g. colorectal carcinomas [116, 120] and SCC [113]. Ln-5 γ2 chain expression has also been demonstrated as an indicator of microinvasiveness in cervical carcinoma [114], and pancreatic carcinoma [171]. In our study we used a monoclonal antibody against y2-chain of ln-5.

Ln5 γ2 chain expression was reported to indicate the invasive potential of precancerous lesions of the anogenital tract [115]. Similar results have been reported in cancer in situ lesions of the larynx [172]. Thus, ln-5γ 2 chain expression seems to be up-regulated in most invasive tumours especially squamous malignancies but down-regulation has been reported in prostate [173] and breast carcinomas [174, 175].

It is known that there is a correlation between ln-5y2 chain expression and the survival in various malignancies such as vagina tongue, lung and anal cancer [119, 176-178]. HPV negativity and high expression of ln-5y2 chain were associated with poor outcome. In a study of 89 cervical cancer including both squamous and adenocarcinoma no correlation of ln-5y2 chain and survival was found, and no difference concerning ln-5y2-chain expression between HPV- positive and HPV-negative tumours [175]. Furthermore, 98% and 69% of the tumours respectively were HPV-positive in contrast to our 34%.

4.4.2 Ki 67 (MIB 1)

Ki-67 expression was evaluated in 70 tumours and all were positively stained. The expression was low, moderate and high in 40 (57.1%), 24 (34.3%) and 6 (8.6%) respectively. Ki-67(MIB1) was significantly correlated to HPV ($p<0.001$), which means that Ki-67 was over expressed in HPV-positive tumours.

This is in agreement with Hoevenaar's et al study on usual and differentiated VIN-lesions; all 38 cases of HPV-positive usual VIN lesions showed high expression of MIB-1 in contrast to 3 of 75 HPV-negative differentiated VIN [179].

In paper II we proposed a better prognosis for HPV-positive tumours compared with HPV- negative tumours [101]. This is consistent with this study where HPV status showed significant impact on survival in a univariate analysis as well as in a stepwise Cox regression analysis.

4.4.3 DNA ploidy and SPF

DNA ploidy and SPF were analyzed in 67 patients. The majority, 50 (74.6%) of the tumours were diploid and 17 (25.4%) tumours were aneuploid. SPF, was low (<10%) in 56 (83.6%) and only 11(16.4%) tumours had a high (>10%) SPF.

The majority of the tumours had a low (<10%) SPF. No correlation was found between ploidy and survival, which is in accordance with previous investigations [19, 180] except for one study there a significant correlation was found in stage I and II but not in stage III and IV [181].

4.5 PROTEOMIC ANALYSIS

We describe a workflow in which tumour protein levels determined by mass spectrometry based proteomics was analysed by two data mining approaches:

- Group wise comparison of altered protein between HPV infected and negative tumours and relapse versus relapse-free tumours and
- Pathway profiling for each individual tumour based on altered protein levels on the hypothesis that individual molecular networks drive tumour growth in each tumour. Comparison of pathways between samples was possible by analysing the ranking for each pathway-match by a supervised multivariate analysis. The aim was to identify key pathways involved in VSCC without losing information as may be the case in a group analysis limited to overlapping proteins.

By using MAYU with a FDR of 5%, 4065 protein entries were considered accurate in terms of identification. Out of these, 1579 were accurately quantified according to PQPQ [182], 449 proteins were present in all 14 samples. They were compared to the mean of the internal standard and were used for the multivariate discriminant analysis and the group wise pathway analysis. A total of 1566 protein entries were included.

4.5.1 Classification based on HPV and relapse status

Student´s t-test identified 4 differently expressed proteins: *Collagen type I alpha 2 and alpha 1; periostin osteoblast specific factor and fibrillin 1* as significantly up-regulated in HPV- positive tumours at an FDR of 22%.

We also sought the protein pattern that could discriminate between relapse and non-relapse cancer groups, irrespective of HPV status. Four proteins were significantly up-regulated in relapse (n=5) compared to the non-relapse patient group (n=7) by student´s t-test at an FDR of 27%. These proteins were: *tryptophanyl-tRNA synthetase* (WARS), *signal transducer and activator of transcription 1* 91kDa (STAT1), *2´-5´-oligoadenylatcyclase* (2´-5´-OAS) and *myxovirus (influenza virus) resistance 1 interferon-inducible protein p78* (MX1). The OPLS-model based on 16 proteins (p-value <0.007), confirmed WARS, STAT1, 2´-5´-OAS and MX1 as important classifiers of relapse and non-relapse tumours. There were no overlapping proteins between the two OPLS models based on HPV status and relapse status.

4.5.2 Biological interpretation of discriminating proteins

The proteins supposed to discriminating HPV-status and relapse/nonrelapse were analysed using Ingenuity Pathway analysis. They were matched against a database consisting of known protein signalling pathways. HPV-status gave 28 significant pathways ($p<0.05$); of which 10 had a p-value <0.01. The top four were α-adrenergic signalling ($p=0.00007$), Protein Kinase A signalling ($p=0.0001$), caveolar mediated endocytosis signalling ($p=0.0009$) and breast cancer regulation by stathmin ($p=0.001$).

The analysis of "relapse/non-relapse" revealed 11 significantly altered pathways ($p<0.05$). The most significant was the *IFN signalling pathway* ($p=0.0005$), followed by *ERK/MAPK signalling, IL-22 signalling* and the protein *ubiquitination pathway* (all with $p=0.03$).

4.5.3 Analysis on individual tumour level

Tumour growth driving pathways are likely to form unique combinations in individual tumours. Pathway analysis was performed for each of the 14 tumour samples individually to obtain altered pathway fingerprints of each patient based on proteomics data. All proteins found to be up- or down regulated were included in the individual tumour analysis; in total 1566 protein entries. The protein profile from each tumour was mapped to the canonical pathways in the Ingenuity database.
The canonical pathway analysis identified the pathways from the IPA library that were most significant for each of the 14 data sets. Individual pathway analysis and following multivariate analysis were done with HPV and relapse as class-identifiers. The protein level alterations in pathway "*RIG-1 like receptors in antiviral innate immunity*" was top ranked.

The investigation of *relapse versus non-relapse* on individual tumours (pathway analysis combined with multivariate analysis) identified *"Rac signaling"* as a top affected pathway in non-relapse tumours, and *"RIG-1 like receptors in antiviral innate immunity"* as top affected pathway in relapse cases. Rac is member of the Rho family of small GTP-binding proteins that mediate ErbB responses. Rac is overexpressed in oral SCC [193] and overexpression correlates with progression of

breast [194] and gastric cancer [195]. RAC1 is activated in breast cancer cell lines in response to the ERBB ligands epidermal growth factor (EGF) and heregulin [196]. The latter study and another [197] indicates that via a protein Rex1, signals are integrated from both tyrosine kinase receptors and GPCRs to activate RAC activity, resulting in the proliferation and migration of breast cancer cells. Rac inhibition impairs breast cancer cell motility and proliferation in response to EGFR and ErbB3 ligands [198, 199]. The activity of Rac is mainly regulated by guanine nucleotide inhibitors and exchange factors.

The RIG1-related pathway is shown to be deregulated in relapse cases. The proteins FADD (Fas-associated death domain) and NF-κB that were mapped to the pathway are linked to poor outcome in lung adenocarcinoma. Increased levels of phosphorylated FADD leads to an increase of NF-κB activity, which in turn affects cell cycle progression [200].

Significant pathways differing between HPV-positive and HPV-negative cases were "*purine metabolism*" and "*glucocorticoid receptor signalling*" (active in HPV) and "*integrin-linked kinase*" (ILK) (active in HPV-negative). Note that the glucocorticoid signalling pathway overlaps both with EGF and with STAT signalling which is part of the interferon pathway connected to viral infections.

4.5.4 IHC staining of selected proteins

We selected 4 proteins that were found to be deregulated in relapse VSCC (both HPV-positive and negative cases) for additional analysis by IHC. These were PSMA5, STAT1, MX1 and LGMN. Staining of HPV-negative tumours was significantly stronger for all proteins ($p<0.05$) in comparison to HPV-positive tumours. In contrast to what was assumed based on the mass spectrometry results there were no statistically significant differences observed in the IHC data between relapse and non-relapse.

4.5.5 Group wise analysis

The protein PSMA5 (identified in the individual tumour analysis as well as a discriminator of relapse versus non-relapse) is a part of the proteasomal unit which belongs to the protein ubiquitination pathway which was one of the top

discriminating pathways in the individual tumour analysis for both relapse and HPV status. The proteasomal unit is reported as up-regulated in many cancer cells, most likely because cancer cells have a need to get rid of immature and irregular proteins which would otherwise harm the cell. Proteasome inhibitors are approved for clinical use in certain cancer types (e.g multiple myeloma), but not in VSCC [183]. The IHC results showed no significance between relapse/nonrelapse patient groups, but in the comparison of HPV-positive tumours with negative, there was significantly ($p<0.04$) lower levels of PSMA5. It is possible that HPV-infected tumours have down-regulation of the proteasomal pathway responsible for viral antigen peptide presentation on MHC1 complex via PSMA5, hence highlighted as difference between these two tumour groups.
This result can then justify further studies on possible therapeutic use of proteosomal inhibitors in HPV-negative VSCC.

One major pathway identified was the interferon (IFN) signalling pathway.
Interferons are activated in response to viral infection, and it has been shown that HPV infection represses the transcription of proteins downstream IFN-signalling [26]. In line with this, we observed lower levels of STAT 1, a key transcription factor in the IFN response, in the HPV-positive tumours in our IHC assay ($p<0.05$), figure 6. This finding was supported in the individual-tumour analysis which identified the IFN pathway as one of the top-deregulated pathways among HPV-positive cases (figure 4). Our findings now confirm the role of IFN pathway in HPV on a protein level. Interestingly, three proteins of the IFN pathway were also identified as most important discriminators of *relapse* in the group-wise analysis on overlapping proteins; MX 1, STAT 1 and 2´5´OAS. These proteins are all IFN-inducible genes identical to those previously identified by microarray studied to be transcriptionally repressed by high-risk HPV proteins [26].

There are also overlaps between several of the pathways identified in this study. STAT signalling is also part of the glucocorticoid pathway as well as the EGFR signalling pathway, which is believed to be altered in some subsets of vulvar carcinoma. Increased expression of HER-2 and EGFR has been found in 47% and 67% of vulvar carcinomas [184-186]. Oonk et al have found that EGFR expression is related to lymph node metastasis in patients with vulvar carcinoma [187].

The role of legumain (LGMN) in VSCC is unclear. It is a lysosomal protease (asparaginyl endopeptidase) involved in class II MHC antigen processing [188]. The pathway-belonging is unknown, but has been suggested as a prognostic factor in breast cancer [189], where positive vesicular staining in IHC was correlated with an adverse outcome. It has also been correlated to invasion and metastasis in solid tumours, and has been suggested as a pro-drug activator [190]. In our study, it was identified as a discriminator of relapse/non relapse (up-regulated in relapse) in the analysis based on overlapping proteins; but as an HPV/non-HPV discriminator in the IHC analysis, showing significantly lower ($p<0.01$) levels in HPV+ compared to HPV- tumours. A link to HPV status is the viral protein E5 which targets and acidifies endosomes, affecting antigen processing [191]. E5 has also been shown to reduce levels of MHC I at the surface possibly by interacting with BAP31 [26, 192], contributing to HPV-mediated immune evasion.
Peptides are however processed differently in the MHC I and MHC II pathways.

In our study, we could see an overlap between pathways discriminating HPV/non relapse and non-HPV/relapse. Several studies have indicated that patients with HPV-positive tumours have a better prognosis (paper II). As the HPV status of a tumour is readily determined by PCR, the extra value of a protein marker for HPV-like phenotype would be showing, not the presence of HPV but more importantly where the HPV infection or residual E5 or E7 protein expression is plays a role in carcinogenesis leading to distinct tumour phenotype. In a study on Head and Neck Squamous Cell carcinoma (HNSCC), Weinberger et al [201] could see no predictive indication from HPV status alone; but when adding information of p16 levels protein downstream E2F, strongly correlated with HPV infection. In a retrospective study from Norway they found that low levels of p16/INK4A protein and high levels of p21 protein were associated with a shorter disease-related survival [202]. HPV-status is unknown, but they found that the patients with high expression of p16/INK4A were younger, indicating the possibility of being HPV-positive. Moreover, a Danish study on HNSCC showed that expression of p16/INK4A, was a prognostic factor of treatment (radiotherapy) response and survival [203].

5 GENERAL CONCLUSIONS

The lymphoscintigram helps to identify and localize the SNs, but cannot determine if unilateral or bilateral groins should be explored. In case of a midline tumour with unilateral radioactive uptake a complete inguinofemoral lymphadenectomy in the side without SN identification should be performed. To optimize detection of SN and minimize the false negative detection rate the method is not recommended for tumours larger than 40mm.

VSCC develops through two different pathways. The differences in tumour size, age and survival between patients with HPV-positive and negative tumours, as well as the presence of HPV DNA in the SN of HPV-positive primary tumours, supports this. In agreement with other studies, HPV types 16 and 18 were dominant in VSCC in our study. The newly introduced HPV vaccines may, in the future, reduce the numbers of VSCC associated with HPV.

Ln-5 y2 chain and HPV status had significant impact on survival in vulvar carcinoma in a univariate analyse. HPV negativity and high expression of ln-5 y2 chain were associated with poor outcome. When considering other clinical and histopathological factors showing impact on survival and including them in a stepwise logistic Cox regression analysis, only HPV status and tumour stage were significant factors for survival.

Alterations of the “RIG-1 like receptors in antiviral innate immunity” pathway may be linked to an unfavourable prognosis while alterations of the IFN/EGFR/Glucocorticoid signalling pathway is associated with HPV-positive tumours and thus of favourable prognosis. Validation with Tissue Micro Array (TMA) on a larger patient cohort is of course needed.

This approach of analysing proteomics data the context of altered pathways may hence help to relate proteome data to upcoming targeted cancer therapies by revealing combinations of tumour growth driving pathways and molecular networks.

6 FUTURE PERSPECTIVES

- Investigating the prevalence of HPV in VSCC over time in Stockholm 1970-2010 . There are reports of increasing numbers of HPV associated VSCC in other countries
- Validation with TMA on a larger patient cohort, the findings of altered pathways and protein expression from paper IV.
- Studies of proteins of importance for malignant transformation, and tumour growth in VIN in relation to normal vulva and invasive cancer. Possibility of identifying patients with VIN III lesions without invasive potential can prevent over-treatment and save many women from mutilating surgery.
- Examine if the de regulated proteins in the tumours can be found in serum or plasma preoperatively and during follow-up. This could help finding early recurrences.
- Investigating the possible effect of proteosomal inhibitors as adjuvant treatment in HPV-negative VSCC.

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