IMMUNOLOGY, IMAGING AND TREATMENT OF ADVANCED PENILE CARCINOMA

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Colofon

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IK ZIE, IK ZIE..

Vincent van der Noort – Wiskundige en kankeronderzoeker

Elke dinsdagmiddag komt een vlotte jonge vrouw mijn kamer binnen om met mijn collega te overleggen over het afhakken, invriezen en in plakjes snijden van menselijke penissen. De vraag is welke (daardoor verkregen) informatie voorspellend is voor de overleving van peniskankerpatiënten. Ik werp wel eens een steelse blik op de staafdiagrammen op het computerscherm van mijn collega. Onder de factoren die op een langere overleving wijzen steekt er één met kop en schouders bovenuit: de aanwezigheid van HPV (Humaan Papillomavirus).

Betekent dit dat we massaal de condooms aan de wilgen moeten hangen en het veelbesproken vaccinatieprogramma voor meisjes moeten opschorten? Natuurlijk niet. Dit ogenschijnlijk gunstige effect van HPV is niet de verdienste van het virus. In een (figuurlijke) doorsnee van alle Nederlandse mannen leven mannen met HPV gemiddeld juist korter. Maar de mannen in het onderzoek hebben allemaal kanker, anders waren ze nooit in onze database terecht gekomen. Bij velen is HPV de oorzaak van de ziekte, maar de mannen die zonder HPV toch kanker hebben, hebben vaak een veel agressievere variant. Achteraf logisch, maar toch is er altijd die ene seconde waarin je denkt een Wereldschokkende Ontdekking te hebben gedaan.

Dit is een voorbeeld van selectiebias, een altijd op de loer liggende denkfout. In mijn geval duurde de verwarring niet lang, maar dat is niet altijd het geval.

In de Eerste Wereldoorlog werden van het front teruggekeerde vliegtuigen met staalplaten versterkt op de plekken waar kogelgaten zaten. Pas vele dode piloten later opperde iemand dat juist de plekken waar ze niet geraakt waren versterkt moesten worden. Dáár zaten waarschijnlijk de kogels in de vliegtuigen die niet terugkeerden.

Waarom is deze denkfout toch zo hardnekkig? Omdat je jezelf er steeds aan moet herinneren dat de groepen die je niet ziet (mannen zonder peniscarcinoom, neergeschoten vliegtuigen) echt bestaan. Dat klinkt makkelijk. Maar denk aan het spelletje 'ik zie, ik zie wat jij niet ziet' en de ander begint als een gek dingen op te noemen die hij wel ziet. Mensen zijn onverbeterlijk.

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Chapter 1

Introduction and Outline

INTRODUCTION AND OUTLINE

In part adapted from Textbook of Penile Cancer[1], chapters *Epidemiology of penile cancer*, *Etiology of penile cancer*, *Management of Lymph Nodes*, and *Follow-Up of Patients with Penile Cancer.*

Penile cancer is a rare malignancy and a potentially mutilating disease for men. The majority of tumours are squamous cell carcinomas (SCC) arising from the glans or inner prepuce.[2] It may appear as a wound-like lesion that does not heal, but in case of a narrow foreskin can be obscured. The patient may notice thickening of the skin or a foul-smelling discharge.

Roughly, for penile cancers two distinct pathogenic pathways are identified.[3,4] The first is related to high risk human papilloma virus (hrHPV) infection, the second is related to chronic irritation and inflammation, for example due to lichen sclerosus (LS) and phimosis.

Penile cancer usually shows a typically step-wise lymphogenic spread prior to hematogenic dissemination. The primary draining lymph nodes are almost invariably located within the inguinal region. Thereafter, dissemination usually continues to the pelvic nodes and/or distant sites. At initial presentation, distant metastases are present in only 1–2% of the patients and are virtually always associated with clinically evident lymph node metastases.

Most patients present with no metastases and have a good prognosis. But presence of lymph node metastases is the single most important prognostic factor, as with stepwise spread of metastatic disease into lymph nodes and then the distant organs, the long term prognosis is considerably reduced.[5–7] The cornerstone of treatment is surgery, but for extensive lymph node metastases multimodality treatment is indicated, and for palliation mostly non-surgical options are used.

Epidemiology
In Western Europe and the United States the age adjusted incidence of penile cancer is 0.7–2.1 per 100,000, accounting for approximately 0.2 % of all male malignancies in Western countries.[8–10] Penile cancer incidence varies widely and can account for over 10 % of malignant disease in men in certain regions across the world, with the highest incidence reported in Romania and Brazil (>6/100.000) and the lowest in Israel (0.1/100.000).[11–13]

The substantial worldwide variation in penile cancer incidences is most likely due to the differences in socio-economic conditions and cultural practices such as circumcision. Penile cancer is rarely seen in populations who routinely practice circumcision during the neonatal or childhood period.[14–17]

Aetiology and risk factors

About 25–50% of penile cancers are linked to oncogenic (high risk) types of the human papilloma virus (hrHPV) which appears to be a favourable prognostic factor.[4,18,19]

Furthermore, genital lichen sclerosus (LS) in men is a chronic inflammatory condition which can result in the development of a phimosis. LS is associated with up to 30 % of penile carcinomas. Other factors associated with an increased risk of penile carcinoma are tobacco use, non-circumcision and HIV infection.

HPV

In systematic reviews, 40–51% of penile cancers are hrHPV-associated, with HPV-16 being the predominant viral subtype.[17,20,21] The majority of cancers is squamous cell carcinoma (SCC) of the usual histological subtype. Similar to vulvar and head and neck carcinomas, SCC of the basaloid and warty subtype display the strongest association with hrHPV (ranging from 66 to 100%) and their etiological relationship with hrHPV infection is most plausible.[22,23]

HPV can be transmitted via sexual contact, but also non-sexually.[24] A persistent infection with hrHPV can be the start of malignant transformation, although in uterine cervical infections only <5% of persistent infections lead to a malignancy. Overexpression of the viral oncogenes E6 and E7 leads to deregulation of cell cycle controlling pathways, as E6 and E7 bind to and inactivate p53 and Rb. This leads to more genomic instability, more (epi)genetic changes and ultimately, to malignant transformation.[25]

Lichen Sclerosus

HPV-negative tumours are thought to arise from a background of chronic inflammation and/or irritation, such as Lichen Sclerosus (LS). The exact etiopathology of LS is unknown, but genetic factors, autoimmune mechanisms and irritative (urine) or infective influences have been postulated as being causal.[26] LS is characterized by chronic inflammation and atrophy and clinical evaluation shows areas of pale atrophic and sclerotic plaques (patches). Its course is progressive and plaques may fuse and become sclerotic, sometimes

resulting in meatal stenosis, circumcoronal adhesions and significant phimosis.[27] Observational studies show that LS is frequently present in peritumoral tissue thus establishing a rationale that LS plays a role in the development of penile carcinoma. Scarce evidence suggest a transformation rate of 2–10 %.[28-30]

A complicating factor in describing a (potential) relationship is the lack of evidence showing histologically proven LS incidence numbers in the healthy population. Moreover, the eventual development of phimosis might partially explain the relationship between LS and penile cancer. Interestingly Bunker et al. described no cases of malignant transformation in 329 male genital LS patients, suggesting that an adequate recognition and treatment averts development of invasive carcinoma.[31]

An Austrian study found histopathological evidence for LS in 74% of tissue samples and Lichen Planus (LP) in 26% of HPV[-], together with a dense T-cell dominant lymphocytic infiltrate in almost 50%. LP, likewise, is an inflammatory condition with an unknown aetiology. The authors suggest an enhanced tolerance in the tumour micro-environment (TME) by T-cells that mediate both chronic inflammatory diseases.[32]

Non-circumcision
Non-circumcision is the most important risk factor for penile cancer. The positive effect of circumcision is explained in view of both pathways. First, by preventing conditions such as poor penile hygiene, smegma retention, and phimosis, which have been reported as risk factors for penile cancer.[14,17,33] Phimosis invariably leads to retention of the normally desquamated epidermal cells and urinary products (smegma) resulting in the development of an environment where chronic irritation and bacterial infection cause inflammation of the prepuce and the glans. When performing statistical analyses after exclusion of phimosis as a risk factor, the presence of a foreskin did not increase the risk of penile cancer.[33,34] Although carcinogenesis has been attributed to chronic inflammation due to the irritating effects of smegma, to date no carcinogenic agent has been identified and isolated within smegma itself nor has the carcinogenic effect of smegma been proven.[35-37]

Secondly, circumcision is believed to reduce the risk of HPV-infection by removal of the inner prepuce which acts as a receptive site of infection by the human papilloma virus.[38] The practice of circumcision as a preventive measure still remains controversial with its benefits, complication risks and ethical aspects.[39,40]

Tobacco use

Smoking shows a dose-dependent association with penile cancer. The use of tobacco in any form is a risk factor for penile cancer and has been described in several studies.[14,33,41] Maden et al. found an increased risk of penile cancer in current cigarette smokers which was further related to the number of pack-years. The risk of penile cancer among men who smoked at diagnosis was 2.8 times that of men who never smoked.[14] Similarly chewing tobacco is a significant risk factor for penile cancer and a combination of cigarette smoking and chewing tobacco carries an even higher risk.[42]

Although the association has been repeatedly observed, the exact role that smoking plays in the development of this disease is still not known. Tobacco may act either through one of its metabolites or may act directly after systemic absorption which can alter the patient's immune response.[42]

Socioeconomic status

Among other malignancies, penile cancer showed a significant association with area poverty in a large population based study in the United States.[43] Scandinavian studies found associations between higher stages of penile cancer and low educational level or low disposable income, and between living in poor socio-economic circumstances and poor prognosis. The same accounts for unmarried or single-living men.[44,45]

Other risk factors

The risk of penile cancer in men with a history of **genital warts** due to low-risk HPV infection, has been reported as 8.2 times that of men who have no history of warts.[46] Similarly there is an eightfold increased risk of penile cancer in patients with *HIV*.[47] The increased risk of penile cancer amongst men infected with HIV may be related to the higher incidence of HPV among men with HIV and a lower ability to clear HPV.[47,48] Poblet et al., have suggested that HIV-1 could synergize with HPV, resulting in progression of premalignant penile lesions into invasive cancer.[49] Circumcision reduces the risk of HIV transmission by reduction of the susceptible area and allowing a period of eukeratinisation.[50]

Like phimosis, **poor hygiene** is thought to contribute to chronic irritation, smegma retention and carcinogenesis. Factors leading to poor hygiene such as self-neglect, obesity and a buried penis are also possible risk factors.[51,52]

Patients with psoriasis undergoing treatment with **psoralen-UV-A photo-chemotherapy** are also at an increased risk of penile cancer.[53,54] Maden et al., found that a history of small tears or small abrasions to the penis was associated with a risk of 3.9 relative to men without such a history.[14]

Immune surveillance

Our immune system protects us from infection with bacteria and viruses, but also from cancer cells. This system, where mutated or infected cells are recognized and killed, is called immune surveillance. Cancer patients suffer from tumours that have escaped these surveillance mechanisms.

Malignant cells use a plethora of mechanisms to escape the immune system. These mechanisms include: expression of co-inhibitory factors, expression of non-classical human leukocyte antigens (HLA), downregulated expression of classical HLA, release of cytokines that inhibit T-cell response directly or indirectly, and many others.

Normally, all nucleated cells express peptides (antigens) presented on their cell surface by the HLA complex. In this way, T-cells can recognise the body cells as "self/normal" or "foreign/abnormal" and in case of the latter, generate a T-cell response. Recognition is avoided by downregulation of classical HLA, or expression of non-classical HLA.[55,56]

Checkpoint molecules can provide an inhibitory signal to the T-cell when these ligands bind to the complementary T-cell receptor. Well known examples of co-inhibitory checkpoint molecules are PD-L1, its binding PD-1 and CTLA-4, immunotherapy has been approved blocking these molecules.[57]
Myeloid cells (including macrophages and dendritic cells) are a heterogeneous cell population with an at least equally heterogeneous spectrum of functions in the tumour microenvironment. Populations of myeloid cells have been associated with promoting tumour growth, tumour spread, angiogenesis, therapy resistance and immune inhibition. At the same time, myeloid cells can aid tumour rejection. A lot of these immune escape mechanisms are yet poorly understood.[58,59]

Staging and imaging

The presence of nodal involvement is the single most important prognostic factor in penile cancer.[5,7,60-65] As the currently available non-invasive staging modalities have a low sensitivity in detecting the regional lymph node status (i.e. missing micrometastatic disease), the optimal management of clinically node-negative

(cN0) patients has been the subject of debate. Approximately 10-20% of these cN0 patients have occult metastasis. Therefore, invasive staging methods (dynamic sentinel node biopsy, modified lymphadenectomy or radical inguinal lymphadenectomy) can be used in those patients considered to be at risk for occult metastases, so called "risk-adapted approach".[66] While close surveillance may lead to unintentional delay because of outgrowth of occult metastases, elective as well as risk-adapted inguinal lymphadenectomy is considered unnecessary in 80-90% of such cases, because of the absence of metastases.[67,68] Furthermore, lymphadenectomy is associated with a high morbidity rate. Up to 35–70% of patients have short- or long-term complications.[69–72]

The different staging and imaging modalities for penile cancer will be further described in **chapter 4 and 5** of this thesis.

Tumor Node Metastasis (TNM)

In 2017, the Union for International Cancer Control (UICC) TNM staging system was updated. The presence of two inguinal nodes was considered pN1 in the 8th edition, where it was pN2 in the 7th, pN3 remained unchanged as well as clinical staging (**Table 1**).[73] In this thesis, the 7th edition is used in most chapters, as the included patients were staged before 2017. In chapter 8, the 8th edition is used, and patients that were included during the first years, were re-staged according to the last edition.

Table 1: TNM Clinical classification and staging

T – Primary Tumour

	7th edition 2009	Changes is the 8th edition, 2017
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
Tis	Carcinoma in situ	
Ta	Non-invasive verrucous carcinoma	
T1	Tumour invades subepithelial connective tissue	
T1a	Tumour invades subepithelial connective tissue without lymphovascular invasion and is not poorly differentiated	
T1b	Tumour invades subepithelial connective tissue with lymphovascular invasion or is poorly differentiated	
T2	Tumour invades corpus spongiosum/corpora cavernosa	Tumour invades corpus spongiosum with or without invasion of the urethra
T3	Tumour invades urethra	Tumour invades corpus cavernosum with or without invasion of the urethra
T4	Tumour invades other adjacent structures	

N – Regional Lymph Nodes

NX	Regional lymph nodes cannot be assessed
N0	No palpable or visibly enlarged inguinal lymph nodes
N1	Palpable mobile unilateral inguinal lymph node
N2	Palpable mobile multiple or bilateral inguinal lymph nodes
N3	Fixed inguinal nodal mass or pelvic lymphadenopathy unilateral or bilateral

pN – Regional lymph nodes, pathological staging

pNX	Regional lymph nodes cannot be assessed	
pN0	No palpable or visibly enlarged inguinal lymph nodes	
pN1	Single intranodal metastasis	Metastasis in one or two inguinal lymph nodes
pN2	Metastases in multiple or bilateral lymph nodes	Metastasis in more than two unilateral inguinal nodes or bilateral inguinal lymph nodes
pN3	Metastasis in pelvic lymph node(s), unilateral or bilateral or extranodal extension of regional lymph node metastasis	

M – Distant Metastasis

M0	No distant metastasis
M1	Distant metastasis

Stage

Stage 0	Tis	N0	M0
	Ta	N0	M0
Stage I	T1a	N0	M0
Stage IIA	T1b,T2	N0	M0
Stage IIB	T3	N0	M0
Stage IIIA	T1, T2, T3	N1	M0
Stage IIIB	T1, T2, T3	N2	M0
Stage IV	T4	Any N	M0
	Any T	N3	M0
	Any T	Any N	M1

Treatment and survival

Primary tumour

Penile tumours can be treated in various ways although most tumours are treated with surgical removal. For superficial tumours, low risk, or penile intraepithelial

neoplasia (PeIN), laser ablation can be used, usually in combination with biopsies for histopathologic evaluation. During the years, the use of penile sparing surgery (wide local excision, partial or total glansectomy or partial penectomy) has increased. The risk of recurrence is a little higher after penile sparing surgery, but this does not compromise survival and seemingly quality of life is better.[74,75]

Large tumours can be challenging to manage, as surgical removal may require a skin flap reconstruction for wound closure and/or urinary diversion.[76] With ingrowth in adjacent organs such as scrotum, prostate, or pubic bone the prognosis is poor. Whether these patients should be treated with curative or palliative intent is often difficult to decide and should be discussed individually in multidisciplinary tumour boards. Combining surgery with induction treatment with chemotherapy and/or radiation may be useful to achieve radicality of resection.

Lymph nodes
Patients with non-palpable lymph nodes undergo minimally invasive staging. Standard procedure in the Netherlands Cancer Institute is ultrasound examination with fine-needle aspiration cytology of suspicious lymph nodes and a dynamic sentinel node biopsy procedure in clinically node negative patients. If either one shows metastatic cells, a completion inguinal lymph node dissection is scheduled.

Patients with palpable inguinal nodal disease, require at least pathologic evaluation of the enlarged lymph nodes. Often, an inguinal lymphadenectomy is immediately performed when the patient is deemed operable. In clinically dubious cases, an ultrasound and sentinel node procedure can be considered as the sentinel node procedure includes thorough palpation of the groin and removal of palpably suspicious nodes.

In patients with clinically suspicious groins, the therapeutic value of regional lymphadenectomy justifies the complications associated with this procedure. Surgery remains the cornerstone of therapy in patients with metastatic disease, with cure in approximately 80% of patients with one or two involved inguinal nodes without extranodal extension (ENE).[7,60–64] When the inguinal region contains two or more tumour-positive lymph nodes or ENE, an ipsilateral prophylactic pelvic lymph node dissection is advised because of a substantial risk of metastasis.[77] Cross-over metastasis to the contralateral pelvic lymph nodes has not been reported. When pelvic lymph nodes appear metastatic on imaging, a pelvic lymph node dissection is performed.

Locoregionally advanced disease and palliative treatment

With large and bulky metastases in the groin or pelvic metastases, surgery alone is not sufficient and guidelines advise multimodal treatment.[77] The most applied combination so far is neoadjuvant or adjuvant chemotherapy with adequate lymphadenectomy for responders. Yet, even with chemotherapy, survival rates are unsatisfactory (1-year overall survival (OS) approx. 50%) and toxicity is high (grade 3-4 events in up to 65% of patients).[78-82] Whether concurrent chemoradiotherapy is an alternative for neoadjuvant chemotherapy, is not yet clear. The optimal role of radiation therapy has been debated in penile cancer due to a lack of robust data.[83,84] Further studies are warranted to optimize the combination and sequence of local and systemic therapies in patients with advanced regional disease.

After involvement of the pelvic lymph nodes, penile cancer spreads to the para-aortic lymph nodes and further to other organs (lung, bone, etc.) and is then considered incurable. Systemic therapy often contains taxane or platinum. Palliation can entail resection of large, painful or ulcerating masses, as spread to distant organs without involvement of lower echelons is very rare (although possible in case of basaloid histology). Also, radiotherapy is an option for symptomatic local or metastatic lesions.

Thesis objective

This thesis aimed to be a starting point of improvement of advanced penile cancer care. Especially in these cases, cure can be hard to obtain and a need is felt for more and better treatment options. Firstly, with Science Magazine calling immunotherapy out as the "Breakthrough of the Year" in 2013, we wanted to assess whether penile cancer bears targets for immunotherapy.

Secondly, we focussed on different imaging modalities, investigating room for improvement. Patients with pelvic metastases do not fare well with surgery alone. Moreover, pelvic metastases are mostly detected upon surgical resection and multimodal treatment is indicated. Early detection enables early start of multimodal treatment strategies that hopefully will lead to higher cure rates. Conventional imaging (CT, MRI) have a sensitivity of only 40% but FDG-PET/CT showed excellent test performances albeit only in small series. We aimed to evaluate FDG-PET/CT ability to detect advanced stages of disease adequately.

Lastly, this research aimed to evaluate therapeutic strategies. Due to the rarity of the disease, and in particular advanced stages, large clinical trials comparing multimodal treatments are almost absent. Patients undergoing large

surgical interventions do not always seem to fare well. A trial with neoadjuvant chemotherapy in our centre had somewhat disappointing results. By setting up a retrospective cohort of patients requiring flap-reconstruction we wanted to quantify challenges in this rare patient category. By setting up a prospective chemoradiation trial, we aimed to evaluate chemoradiotherapy as a treatment alternative for locoregionally advanced penile cancer.

Outline of thesis

In **chapter 2** we investigated expression of the PD-L1 checkpoint molecule in primary penile tumours. In **chapter 3** more of the TME was analysed together with PD-L1 expression: T-cell markers, myeloid markers, HPV status and HLA expression were also included. As clinical outcomes we used presence of lymph node metastases at diagnosis and disease specific survival.

The **chapters 4 and 5** review staging and imaging of penile cancer, with the use of PET/CT scan in particular. **Chapter 6** describes a retrospective cohort where PET/CT was used for staging and evaluated its ability to accurately detect pelvic metastatic lymph nodes and distant metastases before invasive lymph node staging is done.

Chapter 7 is a two-centre evaluation of large surgical procedures using a vascularized muscular flap for reconstruction of the primary or inguinal resection site. Due to the rarity of the locoregionally advanced stages and the procedure only fifteen patients were included.

A multimodality treatment alternative for locoregionally advanced cancer is presented in **chapter 8**. It is a preliminary script of a prospective study looking at curative combined chemotherapy and radiation therapy for advanced penile cancer. **Chapter 9** provides a general discussion of this thesis, **chapter 10** provides a summary in English and Dutch.

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Chapter 2

Expression of programmed death ligand 1 (PD-L1) in penile cancer is of prognostic value and associated with HPV status

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ABSTRACT

Purpose: PD-L1 (Programmed Death Ligand 1) inhibits T-cell function and prevents tumor eradication. This is facilitated by PD-L1 positive tumor cells and PD-L1 positive immune cells, and can be prevented by anti-PD-1 (Programmed Death 1)/PD-L1 immunotherapy. In advanced penile cancer there is a need for new therapeutic strategies. We investigated PD-L1 expression in penile cancers and compared PD-L1 expression with disease-specific survival, lymph-node metastases at diagnosis, and high-risk human papilloma virus (hrHPV) status in a large patient cohort.

Materials & Methods: A total of 213 primary tumors were immunohistochemically stained for PD-L1 and scored for tumor (percentage), stroma (binary) and PD-L1$^+$ tumor infiltrating macrophages (TIM). Additionally, PD-L1$^+$ tumors were scored for expression pattern, that is diffuse or predominantly present at the tumor-stroma margin.

Results: Staining was successful in 200 tumors, of which 75% were hrHPV$^-$. Median follow-up was 62 months. Of 200 tumors, 96 (48%) were PD-L1$^+$ (scored 1% or greater), of which 59 (62%) had a marginal expression pattern and 79 (82%) were hrHPV$^-$ (p = 0.03). Compared to PD-L1$^-$ tumors, the PD-L1 expression patterns each had different prognostic values, in the whole cohort as well as in the hrHPV$^-$ subgroup. On multivariable analyses, a marginal expression pattern was associated with absence of lymph-node metastases (OR 0.4), while diffuse expression was associated with poor survival (HR 2.58). These results were more prominent in the hrHPV$^-$ subgroup (OR 0.25,HR 3.92).

Conclusions: PD-L1 was expressed in 48% of penile carcinomas and mainly in HPV$^-$ tumors. The pattern of expression was a prognostic factor as marginal expression was associated with absence of lymph-node metastases, and a diffuse expression was associated with poor survival.

INTRODUCTION

Penile cancer is a rare but potentially lethal disease with an age adjusted incidence of 0.6-2.1 per 100.000 in Western countries.[1,2] Involvement of regional lymph nodes is the most important prognostic factor as five year survival decreases from greater than 90% to approximately 50% when lymph node metastases are present.[3] Current treatment options for the latter patient group are not always curative and treatment for locally inoperable tumors or distant metastases is almost invariably palliative, indicating the imperative need for new therapeutic options. In various other malignancies, new therapies effectively target tumor interactions with the host immune system.[4,5]

Malignant cells suppress and escape the immune response in various ways, leading to an immunotolerant state in the tumor microenvironment.[6] One of the immunotolerance enhancing checkpoints is the Programmed Death 1 (PD-1)/ Programmed Death Ligand 1 (PD-L1) axis. PD-1 is a cell receptor on T lymphocytes. When PD-L1 binds to PD-1, T cells receive an inhibitory signal.[7] PD-L1 is expressed by tumor cells as well as by immune cells such as macrophages and T cells.[8] Ample expression of PD-L1 has been found in squamous cell carcinomas.[8–10]including dysregulation of immune checkpoints such as the PD-1:PD-L1 pathway. We investigated the expression of programmed cell death-ligand 1 (PD-L1 New therapies block the PD-1/PD-L1 checkpoint, preventing immunotolerance.[8,11] Recently, the first study on PD-L1 expression in penile squamous cell carcinomas showed PD-L1 expression in 62.2% of primary tumors, which correlated with regional lymph node metastases and poor survival.[12]locally-advanced and/or metastatic penile squamous cell carcinoma (SqCC This provides a rationale for anti-PD-1/PD-L1 immunotherapy in patients with penile cancer. However, that study was performed on a limited cohort of 37 patients.

In the current study, we report PD-L1 expression in a large and clinically well-defined cohort 213 patients with penile carcinoma. In addition, we analyzed PD-L1 expression in the context of high risk human papilloma virus (hrHPV) status, lymph node involvement and disease specific survival (DSS).

MATERIALS AND METHODS

Patients

Patients surgically treated for primary penile carcinoma between 2000 and 2009 at the Netherlands Cancer Institute were selected for analysis if they did not receive radiation or chemotherapy prior to surgery, did not receive primary treatment elsewhere and had invasive cancer, excluding carcinoma in situ.[13,14] The use of clinical material was approved by the institutional translational research board. Clinical data on all patients, including TNM classification (2009 modification) and follow-up, were available from a prospectively maintained institutional database. Histopathological data, including HPV status, lymphovascular invasion (LVI) and grade of differentiation, were available from previous studies.[13]

Staining & scoring

From 213 primary tumors, 3 µm whole mount sections of formalin-fixed, paraffin-embedded tissue were immunohistochemically stained for PD-L1 using a BenchMark Ultra autostainer. Paraffin sections were heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep Solution (Ventana Medical Systems, Tucson, Arizona). Heat induced antigen retrieval was performed using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 64 minutes at 95°C. PD-L1 was detected using a rabbit monoclonal antibody (clone E1L3N, Cell Signaling Technology®, 1:200 dilution, for 1 hour at room temperature). Bound antibody was detected using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with hematoxylin.

Staining was scored manually for tumor cells, stroma and tumor associated immune cells. Tumor positivity was scored by two of us (ESJ, SRO) as the percent of positive tumor cells, considering 1% or greater as a tumor positive for PD-L1. This cutoff level was based on published data showing successful anti-PD-1 therapy in patients with low PD-L1 expression.[4] Cytoplasmic staining was ignored because it may represent intracellular stores of PD-L1 and only membranous PD-L1 is thought to be functional.[15] Problematic cases were discussed with an experienced pathologist (JdJ). Additional tumor scoring was done for notable PD-L1 expression patterns, including diffuse – throughout the tumor and marginal – predominantly at the tumor-stroma margin. Immune cells in stroma were then scored binary (negative/positive). Tumor infiltrating macrophage-like cells, referred to as TIMs, were identified by size, shape and position (large and round with dendrites and in tumor fields). PD-L1+ TIMs were also scored binary (absent/present). PD-L1+ myeloid immune cells arranged as a cordon sanitaire around

the tumor, were identified by similar criteria as for TIMs and scored separately (absent/present).

In cases with PD-L1$^+$ TIMs or a PD-L1$^+$ cordon, we analyzed the expression of CD163 (a marker of tumor associated macrophages) on consecutive slides using similar preparation of 3 μm sections, antibody clone MRQ-26 (Cell Marque™) with the ready to use dispenser for 32 minutes at 37°C and the ultraView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with hematoxylin II and bluing reagent (Ventana Medical Systems).

Analysis

PD-L1 expression was compared to hrHPV-status, lymph node status and survival. Statistical analyses were done in SPSS® version 22. Correlations between categorical variables were tested with the 2-sided chi-square test, and correlations with continuous variables were tested with the Spearman correlation test. Hazard ratios (HRs) and multivariable survival analysis were done using Cox regression models, odds ratios (ORs) on multivariable analysis were estimated by logistic regression. Disease specific survival (DSS) was calculated using Kaplan-Meier estimates, with the log rank test used for comparisons. Two-sided p <0.05 was considered statistically significant.

RESULTS

Expression

PD-L1-staining was successful in 200 of 213 tumors **(Figure 1).** A total of 11 sections were uninterpretable due to unreliable staining caused by tissue fixation variability, excess parakeratosis, or lack of tumor cells in the section. **Supplementary Table 1** compares clinicopathological parameters between PD-L1$^+$ and PD-L1$^-$ cases at a cutoff of 1%. We tested multiple commonly used cutoff values including 1%, 5%, 10% and 50%.[8] At the 1% cutoff PD-L1 was positive in 96 tumors (48%) while 28%, 24%, and 7% of cases was scored as 5% or greater, 10% or greater, and 50% or greater, respectively. PD-L1 positivity significantly correlated with the grade of differentiation at all cutoff values (each p < 0.01, **supplementary Table 1).** Correlations with hrHPV and lymph nodes are described below. No other significant correlations were found.

Of 96 positive tumors 59 (61%) had a distinctive marginal expression pattern, predominantly at the tumor-stroma interface (**Figure 1c**).

TIM scoring was possible in 194 tumors with TIMs present in 27.8% (**Figure 2a**).A PD-L1[+] cordon or border was present in 15.7% of tumors (**Figure 2c**). The myeloid origin of PD-L1[+] TIMs and cordons was confirmed by CD163 staining on consecutive slides (**Figure 2b and d**).

Stroma scoring was possible in 198 tumors. Stroma positivity was seen in 150 tumors (75.8%), which strongly correlated with tumor positivity, cordon-positivity and the presence of PD-L1[+] TIMs (each p < 0.01). Only 38 samples (17.8%) had no PD-L1 at all.

HrHPV status

HPV data on this patient cohort, which were previously published, showed a significant survival benefit in hrHPV[+] patients.[13] In short, samples were classified hrHPV[−] or hrHPV[+] using a GP5+/6+ polymerase chain reaction and subsequently genotyped by bead based array on the Luminex® platform.[13]

Of the 200 tumors successfully stained for PD-L1 75.2% were hrHPV[−]. The hrHPV negative cases were significantly more often positive for PD-L1 expression on tumor cells (p = 0.03 see **Table**). This association was not significant at other PD-L1 cutoff values. hrHPV status did not correlate with other PD-L1 parameters or lymph node status (**supplementary Table 2**, and see **Table**).

Lymph node status

Of 66 patients (31%) with tumor positive lymph nodes 6 (9%) had no successful PD-L1 staining, 31 (47%) showed PD-L1[+] status, and 21 (32%) were scored as 5% or greater. Of 147 patients (69%) without lymph node metastases 7 (5%) had no successful PD-L1 staining, 44% were PD-L1[+] and 23% were scored 5% or greater. Of all of the different PD-L1-parameters scored only the PD-L1 tumor cell expression pattern was significantly associated with lymph node status at staging with more lymph node metastases present in cases with diffuse PD-L1 expression (p < 0.01, **supplementary Table 2**).

On multivariable analysis of relevant clinical parameters the grade of differentiation, LVI and pattern of PD-L1 expression remained significant prognostic factors for lymph node metastasis (**supplementary Table 2**). These parameters were included in receiver operating characteristic (ROC) curves to calculate the concordance-index (area under the curve, which is a measure of the proportion of concordantly predicted outcomes). Adding the pattern of PD-L1 expression to this model improved the concordance-index 4% from 77.8 to 81.8.
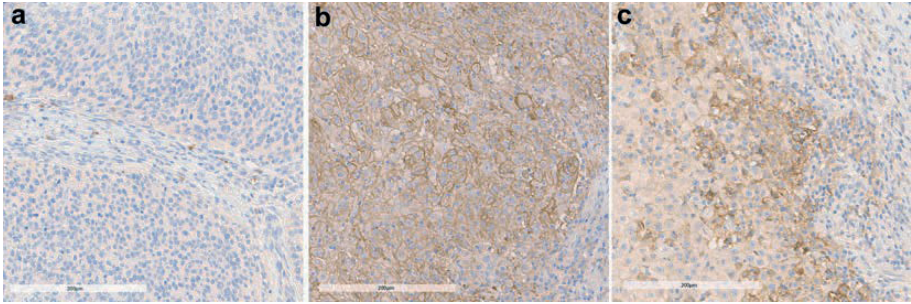
Figure 1. Representative examples of PD-L1 expression show no PD-L1 expression in tumor cells (*a*), and different PD-L1 expression patterns, including diffuse expression (*b*) and expression predominantly at tumor-stroma margin (*c*). Reduced from x20.
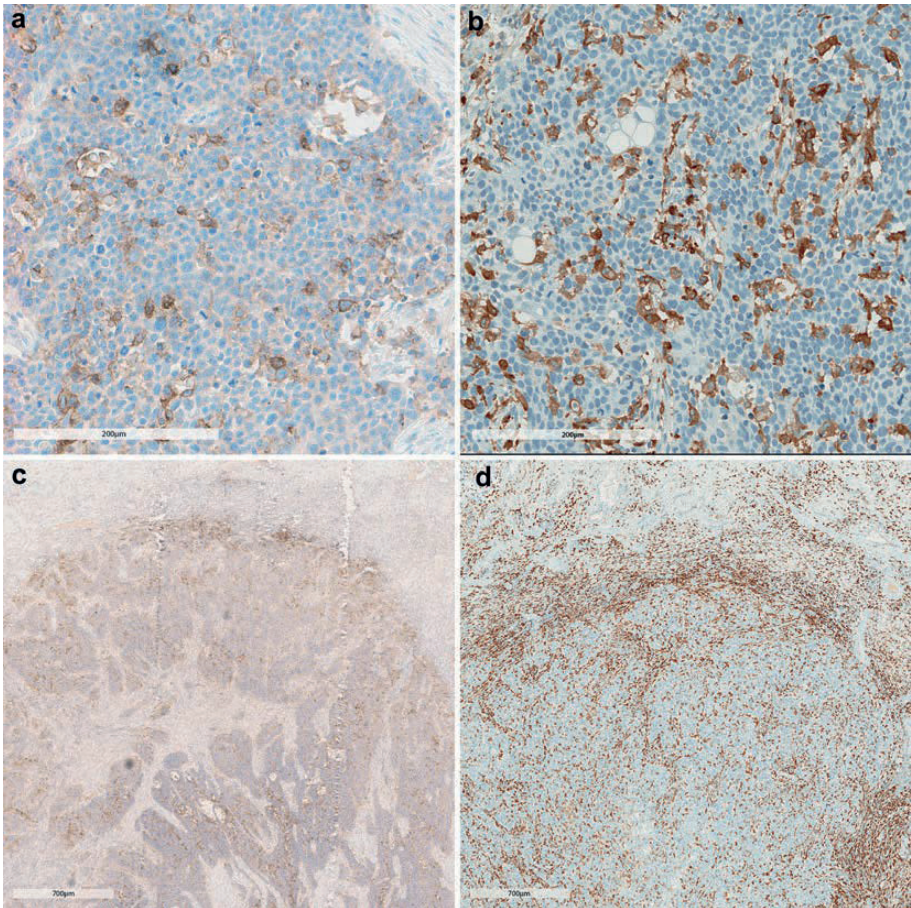
Figure 2. Identification of PD-L1 positive TIMs (*a*) and PD-L1 positive cordon (*c*) compared to CD163 staining of same tumor areas (*b* and *d*, respectively). Reduced from x20 (*a* and *b*) and x3 (*c* and *d*).

Disease Specific Survival

Median follow-up in the current cohort was 62 months. PD-L1 expression of the tumor (positive vs. negative) was not a prognostic factor for DSS at all tested cutoff values (at 1%, 5%, 10% and 50% log rank p = 0.16, 0.87, 0.81 and 0.80, respectively). However, among PD-L1+ tumors a diffuse expression pattern of PD-L1 was associated with a poorer DSS than for tumors with a marginal expression pattern (HR 4.38, 95% CI 1.54 - 12.43, p < 0.01) especially in HPV− cases (HR 6.29, 95% CI 2.03-19.52, p < 0.01, **figure 3**). Univariable survival analysis revealed no prognostic value for the presence of PD-L1+ TIMs, PD-L1+ stroma or a PD-L1+ cordon (**supplementary Table 3**).

Multivariable survival analyses were performed (**supplementary Table 3**). All significant prognostic factors on univariable analysis were used, ie lymph node status, tumor size, grade of differentiation, LVI and hrHPV. We left out pT-stage because it partially represents tumor size, **supplementary Table 3**. On this analysis PD-L1 expression pattern appeared to be an independent predictor for survival compared to PD-L1− tumors (marginal pattern HR 0.45, p = 0.20 and diffuse pattern HR 2.58, p = 0.04). When performed in the hrHPV− subgroup, this prognostic value was even more pronounced (marginal expression 0.31, p = 0.10 and diffuse expression HR 3.92, p = 0.01).

When PD-L1+ TIMs were tested against the same clinicopathological variables, no independent prognostic value was found (HR 0.90, p = 0.83) in the whole population or in the hrHPV− subgroup (data not shown).

DISCUSSION

Inhibition of the PD-1/PD-L1 axis is effective against different tumors such as melanoma and non-small cell lung cancer (NSCLC),[4,16] and promising therapeutic effects have been observed in renal cell and bladder cancer.[17,18] Recently, PD-1/PD-L1-checkpoint inhibitors were approved by the United States FDA (Food and Drug Administration) for advanced stages of melanoma and non-small cell lung cancer. However, to our knowledge no studies have been done to evaluate the clinical effect of PD-L1 inhibitors in penile cancers.

In the current study of a large and clinically well-defined penile carcinoma cohort of 213 patients we found that 48% of the tumors express PD-L1 and that PD-L1 expression was linked to the absence of hrHPV (p = 0.03). When comparing PD-

L1 positive tumors to PD-L1 negative tumors, a marginal expression-pattern (at the tumor-stroma interface) was independently associated with negative lymph node status (OR 0.4) while diffuse expression was independently associated with poor survival (HR 2.58). The presence of PD-L1+ TIMs or a PD-L1+ cordon had no significant association with survival.

Table 2: PD-L1 expression according to hrHPV status

Variable	hrHPV⁻ N=158 (%)	hrHPV⁺ N=52 (%)	Total N=210 (%)	P-value*
Tumor cells				**0.03**
PD-L1+	78 (49.4)	17 (32.7)	95 (45.2)	
PD-L1⁻	70 (44.3)	32 (61.5)	102 (48.6)	
Missing	10 (6.3)	3 (5.8)	13 (6.2)	
Expression pattern				0.79
Diffuse	29 (37.2)	7 (41.2)	36 (37.9)	
Margin	49 (62.8)	10 (58.8)	59 (62.1)	
PD-L1+ TIM				0.19
Present	44 (27.8)	10 (19.2)	54 (25.7)	
Absent	99 (62.7)	38 (73.1)	137 (65.2)	
Unknown	15 (9.5)	4 (7.7)	19 (9.0)	
PD-L1+ cordon				0.27
Present	25 (15.8)	5 (9.6)	30 (14.3)	
Absent	121 (76.6)	43 (82.7)	164 (78.1)	
Unknown	12 (7.6)	4 (7.7)	16 (7.6)	
Stroma				0.22
PD-L1+	114 (72.2)	34 (65.4)	148 (70.5)	
PD-L1⁻	32 (20.3)	15 (28.8)	47 (22.4)	
Unknown	12 (7.6)	3 (5.8)	15 (7.1)	

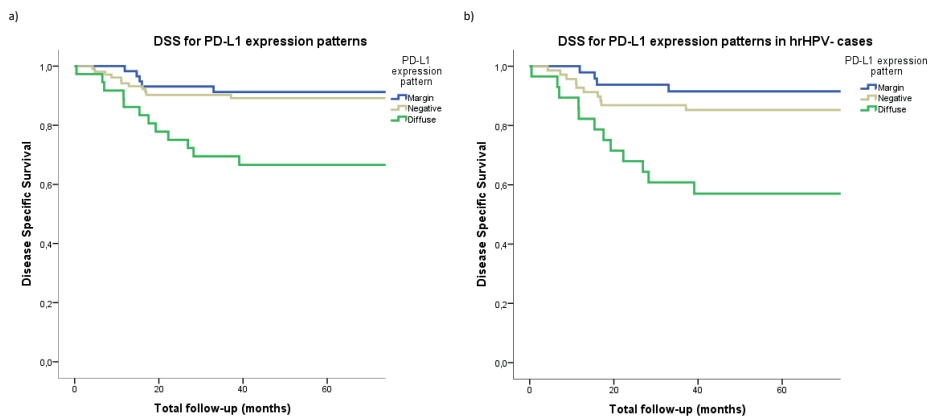* excluding unknown statuses; Chi² test

Figure 3. Kaplan-Meier DSS curves reveal diffuse and marginal expression patterns in whole cohort (log rank p <0.01) (*a*) and in hrHPV negative subgroup (log rank p <0.01) (*b*).

The relatively good prognosis of the cases with a marginal PD-L1 expression-pattern was also seen in cervical cancer.[19] Because PD-L1 can be up-regulated in response to IFN-γ (interferon gamma), we hypothesize that marginal expression is present as a response to the IFN-γ released from activated lymphocytes in the stroma.[6,15,20] In contrast, diffuse expression may be indicative of genetic aberrations leading to PD-L1 expression, as found in melanoma, and cervix and vulva cancer.[21,22] This suggests that marginal positivity reflects an active immune response with corresponding good prognosis and diffuse positivity represent a more genetically altered/poorly differentiated group.

Recently, Udager et al. reported frequent PD-L1 expression in penile cancer.[12] PD-L1 expression on tumor cells was associated with poor survival and positive lymph node status, although the pattern of expression was not considered. In addition, a strong correlation between PD-L1 expression in primary tumors and metastases was reported. We believe that the PD-L1 positive cases described in the latter study were mainly cases with diffuse expression, which is strengthened by the significant correlation observed with poor differentiation. The differences between the results described by Udager et al. and those in our study may be due to different scoring methods as well as to the small sample size and the even smaller HPV[−] subgroups included in their analysis. In addition, the cohort in our study included relatively more tumors of the usual histological subtype (84% compared to 54%).[12]

Expression of PD-L1 has been studied in different cancers, with most investigators using a 1% or 5% cutoff for positivity.[8,23] The 48% incidence of tumor PD-L1 expression in our cohort is higher than in bladder cancer but lower than or comparable to percents reported in melanoma and NSCLC.[8] The prognostic significance of PD-L1 expression on tumor cells seems to differ per tumor type since a worse prognosis in renal cell cancer,[24] gliomas[25] and NSCLC,[26] and a better prognosis in melanoma[27] have been reported. Based on the prognostic value of the marginal and diffuse PD-L1 expression that we observed, we suggest that future studies evaluating the prognostic value of PD-L1 should consider the different expression patterns, especially as we have also observed these differences in cervical cancer.[19] However, substantial response rates to anti-PD-L1 therapy have been reported even in PD-L1[−] tumors.[4] This warrants further investigation on the usefulness of PD-L1 expression as a biomarker in various tumor types.

In bladder cancer PD-L1[+] infiltrating immune cells were present in 27% of tumors, which is a low incidence compared to our cohort.[25] It was suggested that

PD-L1 expression on infiltrating immune cells is more predictive of response to immunotherapy than expression on tumor cells.[11,18,28] In addition, poor prognosis has also been associated with PD-L1[+] immune-cells.[28,29] In contrast to results in a recent study of cervical carcinoma, we found no significant prognostic value for PD-L1[+] TIMs or a PD-L1[+] cordon in the whole cohort or in the HPV negative and positive subgroups.[19]

Some limitations of the current study should be noted. The use of PD-L1 as a prognostic or predictive biomarker is complicated by technical and biological issues.[8] Differences in the choice of antibody, the scoring method, the cutoff value and whether cytoplasmic staining is considered, all result in different outcomes.[8,23] In addition, the identification of staining patterns and using 1% as a cutoff for positivity was arbitrary. However, the clone used in this study has been validated in several studies and the patterns identified were clearly distinguishable.[15,30] In the current series we applied CD163 staining on sequential slides to identify the PD-L1[+] TIMs, and PD-L1[+] macrophages in the cordon and in the stromal compartment. In future studies, multicolor immunohistochemical analysis will allow for the identification of different PD-L1[+] subsets in a single slide.[19] Further, in the current study, we did not analyze PD-L1 expression in lymph node metastases, which may be different from expression levels and patterns in the primary tumor. Therefore, our results do not suggest an effect of immunotherapy on lymph node metastases. Future studies should address this issue. PD-L1 expression had a distinct prognostic value in this study. Although no clinical efficacy of checkpoint immunotherapy can be guaranteed, we believe that these results support the rationale of PD-1/PD-L1 checkpoint immunotherapy effectiveness patients with penile cancer.

Conclusions

This study shows that PD-L1 was expressed in almost half of the penile cancers and mostly in hrHPV[-] tumors. The pattern of PD-L1 expression was a prognostic factor as patients with strong PD-L1 expression at the tumor-stroma interface showed a lower incidence of positive lymph nodes at pathological staging compared to those with PD-L1 negative findings, while a diffuse expression pattern was associated with poor disease specific survival. In the future, it would be interesting to unravel the biological background of the mechanisms leading to these different patterns of PD-L1 expression. Our results support immunotherapy trials targeting the PD-1/PD-L1-checkpoint axis in patients penile cancer.

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Supplementary table 1: Clinicopathological characteristics

Characteristic		PD-L1 ≥1% N= 96 (%)	PD-L1 <1% N=104 (%)	Total N=200 (%)	P-value*
pT-stage	T1a	17 (17.7)	29 (27.9)	46 (23.0)	
	T1b	5 (5.2)	5 (4.8)	10 (5.0)	
	T2	65 (67.7)	60 (57.7)	125 (62.5)	0.40
	T3	8 (8.3)	7 (6.7)	15 (7.5)	
	T4	1 (1.0)	3 (2.9)	4 (2.0)	
Grade	Well	27 (28.1)	48 (46.2)	75 (37.5)	
	Intermediate	44 (45.8)	43 (41.3)	87 (43.5)	
	Poorly	25 (26.0)	12 (11.5)	37 (18.5)	**<0.01**
	Unknown	-	1 (1.0)	1 (0.5)	
Lymphovascular invasion	No	83 (86.5)	85 (81.7)	168 (84.0)	
	Yes	12 (12.5)	18 (17.0)	30 (15.0)	0.34
	Unknown	1 (1.0)	1 (1.0)	2 (1.0)	
Histological subtype	Squamous cell carcinoma	82 (85.4)	86 (82.7)	168 (84.0)	
	Papillary	4 (4.2)	5 (4.8)	9 (4.5)	
	Basaloid	2 (2.1)	3 (2.9)	5 (2.5)	
	Warty	2 (2.1)	3 (2.9)	5 (2.5)	
	Verrucous	-	4 (3.8)	4 (2.0)	
	Mixed basaloid-squamous cell	1 (1.0)	1 (1.0)	2 (1.0)	0.51
	Cuniculatum	1 (1.0)	-	1 (0.5)	
	Sarcomatoid	1 (1.0)	-	1 (0.5)	
	Pseudo-hyperplastic	1 (1.0)	-	1 (0.5)	
	Unknown	2 (2.1)	2 (1.9)	4 (2.0)	
Exophytic growth	No	63 (65.6)	63 (60.6)	126 (63.0)	
	Yes	22 (22.9)	29 (27.9)	51 (25.5)	0.65
	Partially	6 (6.3)	5 (4.8)	11 (5.5)	
	Unknown	5 (5.2)	7 (6.7)	12 (6.0)	
Tumor size	<3 cm	44 (45.8)	48 (46.2)	92 (46.0)	
	≥ 3cm	50 (52.1)	56 (53.8)	106 (53.0)	0.93
	Unknown	2 (2.1)	-	2 (1.0)	
HPV-status	Negative	78 (81.3)	68 (65.4)	146 (73.0)	Overall 0.07;
	Positive low-risk	-	2 (1.9)	2 (1.0)	**hrHPV 0.03;**
	Positive high-risk	16 (16.7)	30 (28.8)	46 (23.0)	lrHPV 0.37
	Positive both	1 (1.0)	2 (1.9)	3 (1.5)	
	Unknown	1 (1.0)	2 (1.9)	3 (1.5)	
Lymph node positivity	No	65 (67.7)	75 (72.1)	140 (70.0)	0.50
	Yes	31 (32.3)	29 (27.9)	60 (30.0)	
Extranodal extension	No	16 (16.7)	22 (21.2)	38 (19.0)	
	Yes	12 (12.5)	6 (5.8)	18 (9.0)	0.09**
	Unknown	3 (3.1)	1 (1.0)	4 (2.0)	
	Negative lymph nodes	65 (67.7)	75 (72.1)	140 (70.0)	

* excluding unknown statuses; Chi² or Fishers exact test
**excluding patients with negative lymph nodes and unknown extension.

Supplementary table 2: Univariable and multivariable logistic regression analyses for lymph node status

Variable	Tested	OR (95% CI)	P-value
Univariable logistic regression analysis			
Tumor size (cm)	Continuous	1.12 (0.998 – 1.28)	0.09
pT-stage	Overall		**<0.01**
1b	Compared to T-stage 1a	5.00 (1.09 – 22.98)	0.04
2	Compared to T-stage 1a	3.71 (1.47 – 9.37)	<0.01
3	Compared to T-stage 1a	7.50 (2.05 – 27.48)	<0.01
4	Compared to T-stage 1a	37.50 (3.72 – 377.73)	<0.01
Grade of differentiation	Overall		**<0.01**
Grade 2	Compared to grade 1	6.19 (2.56 – 14.93)	<0.01
Grade 3	Compared to grade 1	15.99 (5.81 – 44.00)	<0.01
LVI	Present vs. absent	4.19 (1.92 – 9.16)	**<0.01**
HrHPV	Negative vs. positive	1.29 (0.64 – 2.60)	<0.47
PD-L1 tumor (%)	Continuous	1.01 (0.10 – 1.03)	0.18
PD-L1 tumor	≥1% vs. <1%	1.23 (0.67 – 2.26)	0.50
PD-L1 pattern	Diffuse vs. margin	5.13 (2.05 – 12.89)	**<0.01**
PD-L1[+] stroma	Positive vs. negative	0.80 (0.40 – 1.61)	0.54
PD-L1[+] TIM	Present vs. absent	1.70 (0.87 – 3.32)	0.12
PD-L1[+] cordon	Present vs. absent	1.17 (0.51 – 2.67)	0.70
Multivariable logistic regression analysis			
pT-stage	Overall		0.28
1b	Compared to T-stage 1a	1.39 (0.23 – 8.34)	0.72
2	Compared to T-stage 1a	1.82 (0.58 – 5.71)	0.30
3	Compared to T-stage 1a	4.67 (0.98 – 22.36)	0.05
4	Compared to T-stage 1a	7.69 (0.37 – 159.13)	0.19
Grade of differentiation	Overall		**<0.01**
Grade 2	Compared to grade 1	4.48 (1.61 – 12.48)	<0.01
Grade 3	Compared to grade 1	9.97 (2.97 – 33.52)	<0.01
LVI	Present vs. absent	2.78 (1.04 – 7.44)	**0.04**
PD-L1 pattern	Overall		**<0.01**
Diffuse	Compared to PD-L1-	2.11 (0.83 – 5.34)	0.12
Margin	Compared to PD-L1-	0.40 (0.16 – 0.99)	0.05
Multivariable logistic regression analysis in only hrHPV- cases			
pT-stage	Overall		0.40
1b	Compared to T-stage 1a	6.28 (0.39 – 100.10)	0.19
2	Compared to T-stage 1a	1.40 (0.36 – 5.46)	0.63
3	Compared to T-stage 1a	4.14 (0.63 – 26.99)	0.14
4	Compared to T-stage 1a	3.73 (0.10 – 143.25)	0.48
Grade of differentiation	Overall		**<0.01**
Grade 2	Compared to grade 1	9.45 (2.85 – 31.33)	<0.01
Grade 3	Compared to grade 1	13.59 (2.95 – 62.63)	<0.01
LVI	Present vs. absent	5.69 (1.56 – 20.71)	<0.01
PD-L1 pattern	Overall		**0.03**
Diffuse	Compared to PD-L1-	1.10 (0.34 – 3.59)	0.87
Margin	Compared to PD-L1-	0.25 (0.08 – 0.78)	**0.02**
Multivariable ROC analysis		**C-index (95% CI)**	**SE**
T-stage, grade of differentiation and LVI	Without PD-L1 pattern	77.8 (71.0 – 84.5)	3.4
	With PD-L1 pattern	81.8 (75.6 – 88.0)	3.2

CI; confidence interval, ROC; receiver operating characteristic, SE; standard error.

Supplementary table 3: Univariable and multivariable Cox Regression survival analyses

Variable	Tested	HR (95% CI)	P-value
Univariable Cox analysis			
Lymph node status	Positive vs. negative	25.78 (7.79 – 85.30)	**<0.01**
Tumor size (cm)	Continuous	1.20 (1.07 – 1.37)	**<0.01**
pT-stage	Compared to T-stage 1a		
1b		8.95 (1.49 – 53.65)	**0.02**
2		2.81 (0.64 – 12.35)	0.17
3		9.09 (1.76 – 46.84)	**<0.01**
4		49.19 (9.46 – 255.72)	**<0.01**
Grade of differentiation	Compared to grade 1		
Grade 2		2.11 (0.75 – 6.00)	0.16
Grade 3		5.98 (2.11 – 16.97)	**<0.01**
LVI	Present vs. absent	3.25 (1.47 – 7.18)	**<0.01**
HrHPV	Negative vs. positive	4.83 (1.15 – 20.30)	**0.03**
PD-L1 tumor (%)	Continuous	1.00 (0.98 – 1.02)	0.93
PD-L1+ tumor	≥1% vs. <1%	1.71 (0.80 – 3.65)	0.17
PD-L1 pattern	Diffuse vs. margin	4.38 (1.54 – 12.43)	**<0.01**
Diffuse	Compared to PD-L1-	3.39 (1.49 – 7.67)	**<0.01**
Margin	Compared to PD-L1-	0.78 (0.27 – 2.25)	0.65
PD-L1+ stroma	Positive vs. negative	1.36 (0.51 – 3.59)	0.54
PD-L1+ TIM	Present vs. absent	1.73 (0.79 – 3.81)	0.17
PD-L1+ cordon	Present vs. absent	0.87 (0.30 – 2.51)	0.79
Multivariable Cox analysis			
Lymph node status	Positive vs. negative	40.03 (9.60 – 166.88)	**<0.01**
Tumor size (cm)	Continuous	1.30 (1.12 – 1.51)	**<0.01**
Grade of differentiation	Compared to grade 1		
Grade 2		0.33 (0.09 – 1.22)	0.10
Grade 3		1.08 (0.30 – 3.90)	0.91
LVI	Present vs. absent	1.6 (0.62 – 4.33)	0.32
HrHPV	Negative vs. positive	11.30 (2.37 – 53.80)	**<0.01**
PD-L1 pattern	Compared to PD-L1-		
Diffuse		2.58 (1.02 – 6.50)	**0.04**
Margin		0.45 (0.13 – 1.53)	0.20
Multivariable Cox analysis in only hrHPV- cases			
Lymph node status	Positive vs. negative	100.18 (18.26 – 549.69)	**<0.01**
Tumor size (cm)	Continuous	1.42 (1.20 – 1.67)	**<0.01**
Grade of differentiation	Compared to grade 1		
Grade 2		0.20 (0.05 – 0.78)	**0.02**
Grade 3		0.99 (0.28 – 3.50)	0.99
LVI	Positive vs. negative	1.87 (0.62 – 5.67)	0.27
PD-L1 pattern	Compared to PD-L1-		
Diffuse		3.92 (1.46 – 10.52)	**0.01**
Margin		0.31 (0.08 – 1.25)	0.10

3

Chapter 3

The prognostic value of immune factors in the tumor microenvironment of penile squamous cell carcinoma

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ABSTRACT

The host's immune system plays a pivotal role in many tumor types, including squamous cell carcinomas (SCCs). We aim to identify immunological prognosticators for lymph node metastases (LNM) and disease specific survival (DSS) in penile SCC. For this retrospective observational cohort study, penile SCC patients (n = 213) treated in the Netherlands Cancer Institute, were selected if sufficient formalin-fixed paraffin-embedded tumor material was available. Analysis included previously described high-risk human papilloma virus (hrHPV) status, immunohistochemical scores for classical and non-classical human leukocyte antigen (HLA) class I, programmed death ligand-1 (PD-L1) expression, and novel data on tumor-infiltrating macrophages and cytotoxic an regulatory T-cells. Clinicopathological characteristics and extended follow-up were also included. Regression analyses investigated relationships of the immune parameters with LNM and DSS.

In the total cohort, diffuse PD-L1 tumor-cell expression, CD163^{+} macrophage infiltration, non-classical HLA class I upregulation, and low stromal CD8^{+} T-cell infiltration were all associated with LNM. In the multivariable model, only tumor PD-L1 expression remained a significant predictor for LNM (odds ratio (OR) 2.8, p = 0.05). hrHPV negativity and diffuse PD-L1 tumor-cell expression were significantly associated with poor DSS and remained so upon correction for clinical parameters [hazard ratio (HR) 9.7, p < 0.01 and HR 2.8, p = 0.03]. The only immune factor with different expression in HPV[+] and HPV[−] tumors was PD-L1, with higher PD-L1 expression in the latter (p = 0.03).

In the HPV^{−} cohort (n = 158), LNM were associated with diffuse PD-L1 tumor-cell expression, high intratumoral CD163^{+} macrophage infiltration, and low number of stromal CD8^{+} T-cells. The first two parameters were also linked to DSS. In the multivariable regression model, diffuse PD-L1 expression remained significantly unfavorable for DSS (HR 5.0, p < 0.01).

These results emphasize the complexity of the tumor microenvironment in penile cancer and point toward several possible immunotherapy targets. Here described immune factors can aid risk-stratification and should be evaluated in clinical immunotherapy studies to ultimately lead to patient tailored treatment.

INTRODUCTION

Penile squamous cell carcinoma (SCC) is a rare disease with an incidence of less than 1/100,000 in Western countries.[1,2] The prognosis for early stage penile cancer patients is good (5-year survival without lymphogenic spread is 96%) but worsens gradually with presence of lymph node metastases (LNM).[2,3] Surgery is the mainstay of penile cancer treatment, for both primary tumors and LNM. Only in advanced stages (e.g., pelvic lymph node involvement or irresectable disease) multimodal treatment is necessary, mostly in the form of neoadjuvant chemotherapy or adjuvant radiation.[4]

In 20–50% of the patients, penile SCC is induced by a persistent infection with high-risk human papilloma virus (hrHPV).[5,6] Diagnosis, treatment, and follow-up are the same for hrHPV-negative (hrHPV⁻) and hrHPV-positive (hrHPV⁺) tumors.[4] Nevertheless, patients with hrHPV⁺ tumors have a better disease-specific survival (DSS) than patients with hrHPV⁻ tumors (5-year DSS of 96% vs. 82% respectively).[7]

The difference in patient outcomes between hrHPV⁺ and non-virally induced penile cancer may be partially explained by different immune escape mechanisms.[8–14] Surely, immunosuppressive and immunostimulating factors in the tumor microenvironment (TME) co-determine the course of disease in many different cancers, but relatively little is known about penile SCC.[15,16]

For example, in head and neck squamous cell carcinomas (HNSCCs) higher levels of tumor-infiltrating immune cells in hrHPV⁺ tumors are indicated as pivotal role players in a better response to standard therapy in comparison to hrHPV⁻ tumors.[17–19] This concerns high levels of intratumoral CD8⁺ and CD3⁺ T-lymphocytes but also antigen presenting cells such as myeloid dendritic cells.[18–21] CD8⁺ cytotoxic T-cells are capable of immediate tumor-cell killing and therewith are the effectors of anti-tumor response.[21] Regulatory T-cells (Tregs) are well known for their detrimental effect on the immune response.[10,12,22] However, associations of Tregs with clinical outcome remain controversial. High numbers of FoxP3⁺ Tregs were associated with early stage disease and better overall survival in HNSCC, but with adverse patient outcome in colorectal cancer and non-small-cell lung carcinoma.[18,23–25] Cytotoxic and Treg subpopulations have both been described as prognostic factors separately, as well as the ratio between the two.[15,19,20,26] An increased CD8/FoxP3-ratio at diagnosis has been associated with responsiveness to immunotherapy in renal cancer and

melanoma.[15,27–29] Tumor infiltrating macrophages (TIM) are usually macrophages with an immunosuppressive M2-phenotype.[30–32] These macrophages are marked by CD163, and are associated with T-cell response suppression, migration, and treatment evasion.[30,31] High CD163^{+} macrophage infiltration was associated with high disease stage and LNM in hrHPV^{+} cervical cancer, and with poor survival in oral SCC.[32,33]

In penile cancer, various immune escape mechanisms in the TME have been studied (partly by our group).[8–14] In a multivariable analysis by Vasallo et al., presence of FoxP3-positive lymphocytes (presumably Tregs) was associated with poor disease free survival.[10] In addition, a decreased CD8/FoxP3-ratio was associated with tumor progression during follow up.[12] Human leukocyte antigen (HLA) class I was assessed with immunohistochemical (IHC) staining on a tissue microarray (TMA). A prognostic role was only found for HLA-A expression that was associated with decreased overall survival.[9] No differences in HLA expression were observed between HPV[−] and HPV[+] tumors. Programmed death ligand-1 (PD-L1) expression was assessed in multiple studies, using different antibodies and techniques.[10–14] HPV[−] penile cancer cells are more often PD-L1[+]. Tumor cell PD-L1-expression was associated with worse DSS and LNM, especially a diffuse expression of PD-L1 throughout the tumor fields.[11,13,14]

To compare the prognostic value of all these parameters, and to determine which factors have the strongest associations with patient outcomes, different factors from the TME should be evaluated in an integrative analysis. The aim of this study was to gain insight in the TME, and to identify possible associations between TME factors and LNM/DSS in patients with penile cancer.

In this retrospective observational cohort study, we investigated previously determined factors (HPV status, classical and non-classical HLA class I, and PD-L1 expression) in combination with novel data on tumor-infiltrating cytotoxic T-cells, Tregs, and M2-polarized macrophages.[7,9,11]

MATERIALS AND METHODS

Study population and tissue samples
Between 2001 and 2009, 487 consecutive patients were diagnosed with penile SCC in the Netherlands Cancer Institute, Amsterdam. All were considered for inclusion, according to the following criteria. Exclusion criteria were non-invasive

carcinoma, neoadjuvant non-surgical treatment, no tumor tissue available in our institutional biobank (mostly because of surgical removal elsewhere or treatment with laser ablation). Inclusion criterion was that sufficient archived tissue needed to be available in our institutional biobank. Sufficient archived formalin-fixed, paraffin-embedded (FFPE) material was available from 216 patients. All were staged and surgically treated in a standardized way.[34] Clinical follow-up data were updated. Patients were usually clinically followed for 5 years, after that, patient status was sometimes available through municipal administration. This study was carried out with approval of the institutional medical ethical committee that considered this study not falling within the scope of the act of research involving human subjects, it was also approved by the translational research board of our institute.

Evaluation of the IHC stainings on 5 μm sections was performed by two researchers (RSD and ESJ or SRO and ESJ) and an experienced uropathologist (JdJ). Three patients were excluded because a majority of the parameters could not be analyzed (e.g., no invasive tumor present in sample).

HrHPV-typing

For protocols of hrHPV-typing, classical HLA, non-classical HLA and PD-L1 IHC analyses, we refer to our previous reports.[7,9,11] In short, hrHPV-status was determined on 212 tissue samples using GP5+/6+ PCR enzyme-immunoassay for 14 different HPV types.[7]

Immunohistochemistry

A TMA of 168 samples was immunohistochemically analyzed for HLA class I expression with the following antibodies: HCA2 (HLA-A), HC10 (HLA-B/C; both provided by prof. Neefjes of our institute), anti-beta-2-microglobulin (β2m; DAKO, Denmark), MEM-E/02 (HLA-E; Bio-Rad, USA) and 4H84 (HLA-G; from BD Pharmingen, USA).[9] PD-L1 was determined on 213 whole-mount sections using the E1L3N clone (Cell Signaling, USA).[11]

Whole-mount sections from 213 FFPE tissue blocks were immunohisto-chemically stained for CD8 (C8/144B, DAKO, Denmark), FoxP3 (236A/E7, AbCam, England), and CD163 (MRQ-26, Cell Marque, Rocklin, USA) using the Ventana protocol and autostainer with heat induced antigen retrieval. Details of different IHC stainings are summarized in **Table S1** in Supplementary Material.

Immunofluorescent double-staining

Twelve randomly selected cases (six hrHPV[−] and six hrHPV[+] tumors) were double-stained with primary antibodies CD163 (10D6, NCL-CD163, Novocastra, Germany) and CD68 (514H12, MCA1815, Bio-Rad, UK). Secondary antibodies from Life Technologies, USA were used for detection. The slides were analyzed manually using a fully motorized digital imaging fluorescence microscope (Axiovert-200M, Germany). More details of these stainings can be found in **Table S1 in Supplementary Material**.

Scoring methods

Human leukocyte antigen-A, HLA-B/C, and β2m expression were scored in a semiquantitative way with the quality control system proposed by de Ruiter et al. using intensity and percentage, resulting in three categories: negative, weak or positive.[9,35] A combined score of HLA-A, HLA-B/C and β2m grouped tumors into categories of classical HLA class I expression: normal expression (all three positive), complete downregulation (negative β2m or negative HLA-A and HLA-B/C), and partial downregulation (other combinations). Although HLA-A was significant in previous multivariable analysis of this cohort, the total score of classical HLA was used for analysis because it had stronger associations with updated variables (comparative data not shown).[9] HLA-E and HLA-G were scored as absent/upregulated, and a combined score resulted in two groups: tumors into normal expression of non-classical HLA class I (both negative) and upregulation (one or both upregulated).

Only membranous staining of PD-L1 was scored. Percentage of positive cells was noted, cut-off for PD-L1 positivity of tumors was ≥1% of tumor cells.[11,12,36,37] For PD-L1[+] tumors, the tumor expression-pattern was scored as diffuse (throughout the tumor fields) or margin (predominantly at the tumor-stroma margin.[11] Immune cells in stroma were scored binary (negative or positive). PD-L1-positive TIMs were identified by size, shape and position (large, round, with dendrites, and in tumor fields) and were scored as present or absent.[11]

For CD8^{+} and FoxP3^{+} T-cell infiltration analysis, in each sample three peripheral and three central tumor focus fields were randomly selected in Aperio ImageScope (Leica Biosystems, Solms, Germany) and magnified by 20×. Each image (focus field) contained stroma and tumor fields. The number of positive pixels was determined with the semi-automatic computer program Image-J (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Images were deconvoluted with a plug-in to the color red. By setting a threshold (at 180 for every image), the positive pixels

were separated from the negative pixels. For every image tumor fields were digitally selected. The size of the total image area, tumor area and stromal area in pixels was noted, together with the number of positive pixels in these areas. The stromal values were calculated by subtracting the tumor area from the whole image area. In each tumor slide, the average number of positive pixels in the six focus fields was used for both CD8 and FoxP3 in tumor area and stromal area. T-cell ratios were calculated by dividing the CD8$^+$ pixels by FoxP3$^+$ pixels.

Semiquantitative analysis of CD163 in tumor and stroma determined low/high infiltration of CD163$^+$ cells. The 12 immunofluorescently stained samples (CD163/CD68) were qualitatively analyzed.

Statistical analysis

High-risk human papilloma virus subgroups were compared with respect to clinicopathological, tumor and stroma characteristics using chi-square test, Fishers' exact test, and t-tests for independent samples. Also, Kaplan–Meier estimated survival curves were plotted for HPV groups (**Figure 1**). Normality was assessed with Kolmogorov–Smirnov for all continuous parameters. T-cell parameters were transformed to log-scale to meet normality assumption when comparing means (t-test). Pixel counts of CD8 and FoxP3 were divided by 100,000 for statistical analyses so that hazard ratios (HRs) and odds ratios (ORs) represent a substantial change. A constant integer (of 1) was added to stromal CD8 and stroma FoxP3 to prevent division by zero when calculating T-cell ratios. A logistic regression was used to model odds of LNM, and a Cox regression to model DSS from date of diagnosis to death from penile cancer or last follow-up/death from other cause. Characteristics that were significant or nearly significant in univariable models, were considered for final multivariable models found with a backward stepwise selection approach with models comparison using likelihood-ratio tests and $p > 0.10$ as covariate exclusion criterion. All analyses were done using SPSS® version 22 (IBM, Armonk, NY, USA) in collaboration with experienced statisticians (HHT and KJ).
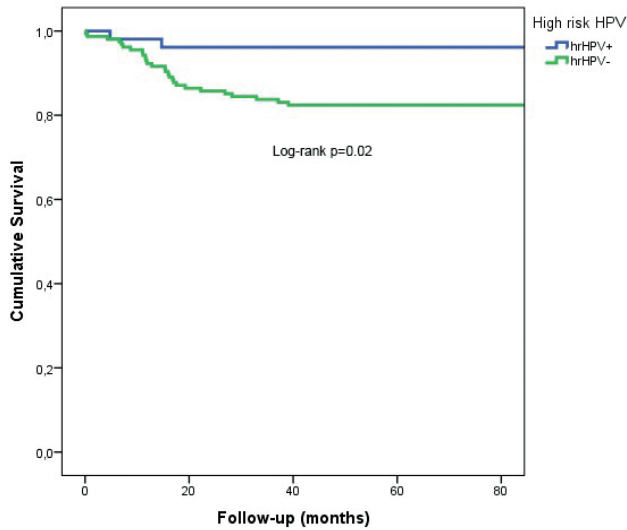
Figure 1. Kaplan–Meier survival plots with log-rank test analysis of high-risk HPV-positive and -negative penile cancer cases.

Baseline	12 months	24 months	36 months	48 months	60 months	
52	52	49	48	48	45	hrHPV$^+$
158	142	130	142	121	115	hrHPV$^-$

RESULTS

Clinicopathological characteristics

In this cohort (n = 213), 68 patients (31.9%) had LNM, and 87 patients (40.8%) died during follow-up; 29 patients (13.6%) died of penile cancer (on average after 14.7 months). Median overall follow-up was 100.7 months (IQR 69.4–119.7). Mean DSS was 166.8 months (median not reached).

Age was normally distributed. Tumor size was not. T-cell parameters (intratumoral and stroma CD8 and FoxP3, and T-cell ratios) were normally distributed after log-scale transformation. Clinicopathological characteristics are summarized in **Table 1**. When comparing the hrHPV subgroups with respect to these characteristics, we observed a significant difference only in differentiation grade ($p < 0.01$) and death by penile cancer ($p = 0.02$). Most well differentiated tumors were hrHPV$^-$ (70 vs. 9 in hrHPV$^+$). Despite this, DSS was better in hrHPV$^+$ patients in comparison to hrHPV$^-$ patients, with 2 and 27 penile cancer related deaths,

Table 1: Clinicopathological characteristics.

Variable	hrHPV⁻, N = 158 (%)	hrHPV⁺, N = 52 (%)	Total, N = 213 (%)[a]	p-Value[b]
Age median (IQR)	67.6 (58.2–74.6)	63.6 (54.4–71.6)	65.9 (57.3–74.4)	0.38
pT stage				0.17
pT1	42 (26.6)	19 (36.5)	61 (28.6)	
pT2	99 (62.7)	28 (53.8)	130 (61.0)	
pT3	11 (7.0)	5 (9.6)	16 (7.5)	
pT4	6 (3.8)	–	6 (2.8)	
Tumor size median (IQR)	3.0 (2.0–4.1)	2.5 (1.5–3.9)	3.0 (2.0–4.0)	0.09
histological subtype				0.08[c]
SCC NOS	137 (87.3)	43 (82.7)	180 (84.5)	
Papillary	8 (5.1)	1 (1.9)	9 (4.2)	
Verrucous	5 (3.2)	–	5 (2.3)	
Warty	2 (1.3)	3 (5.8)	5 (2.3)	
Basaloid	1 (0.6)	4 (7.7)	5 (2.3)	
Mixed SCC-basaloid	1 (0.6)	1 (1.9)	2 (0.9)	
Sarcomatoid	1 (0.6)	–	1 (0.5)	
Cuniculatum	1 (0.6)	–	1 (0.5)	
Pseudohyperplastic	1 (0.6)	–	1 (0.5)	
Missing	1 (0.6)	–	4 (1.9)	
grade of differentiation				<0.01
Well (grade 1)	70 (44.3)	9 (17.3)	80 (37.6)	
Intermediate (grade 2)	62 (39.2)	31 (59.6)	94 (44.1)	
Poor (grade 3)	26 (16.5)	12 (23.1)	38 (17.8)	
Missing	–	–	1 (0.5)	
pn stage				0.84
pN0	107 (67.7)	36 (69.2)	145 (68.1)	
pN+	51 (32.3)	16 (30.8)	68 (31.9)	
extranodal growth				0.12[d]
Present	19 (12.0)	3 (5.8)	22 (10.3)	
Absent	28 (17.7)	13 (25)	42 (19.7)	
No LNM	107 (67.7)	36 (69.2)	145 (68.1)	
Missing	4 (2.5)	–	4 (1.9)	
Death by penile cancer				0.02
No	131 (82.9)	50 (96.2)	184	
Yes	27 (17.1)	2 (3.8)	29	

IQR, interquartile range (25th–75th percentile); SCC, squamous cell carcinoma; NOS, not otherwise specified; HPV, human papilloma virus; LNM, lymph node metastases.
[a]Including three cases with unknown hrHPV status. [b]Excluding missing cases. Comparing the two hrHPV subgroups. Independent sample t-test for continuous variables, chi-square, or Fishers exact test for categorical variables. [c]Divided in SCC NOS, unfavorable subtypes (basaloid, mixed basaloid-warty, and sarcomatoid) and favorable subtypes (papillary, verrucous, warty, cuniculatum, and pseudohyperplastic). [d]Excluding patients with no LNM/unknown lymph node status.
Bold numbers are statistically significant.

respectively (log-rank p = 0.02; **Figure 1**) at mean follow-up of 169.5 vs. 160.5 months. Among hrHPV$^+$ tumors, HPV16 was the predominant type 79% (41/52).[7]

Classical and non-classical HLA expression and PD-L1 expression patterns

Immune characteristics are summarized in **Figure 2** and **Table S2** in Supplementary Material. Aberrant classical and non-classical HLA expression was equally distributed among hrHPV$^-$ subgroups. Interestingly, hrHPV$^-$ tumors were significantly more often PD-L1$^+$ (49.4 vs. 32.7% of hrHPV$^+$; p = 0.03). Also, there was a trend toward hrHPV$^-$ tumors having relatively more of both PD-L1 expression patterns compared with hrHPV$^+$ tumors (p = 0.09).[11]

Tumor-infiltrating cytotoxic T-cells and Tregs

The presence of CD8$^+$ T-cells and FoxP3$^+$ Tregs was determined by standard IHC staining. Representative examples of CD8 and Foxp3 presence are depicted in **Figures 3A–D**. Interestingly, CD8 and FoxP3 pixel counts were much higher in stromal areas than in tumor areas, in both hrHPV$^-$ and hrHPV$^+$ tumors (**Figure 2**). No differences in T-cell numbers or CD8/FoxP3-ratio were found between hrHPV$^+$ and hrHPV$^-$ tumors (**Figure 2**; **Table S2** in Supplementary Material).

Tumor-infiltrating macrophages

Representative examples of CD163 IHC stainings are depicted in **Figures 3E,F**. No significant differences in CD163$^+$ macrophage intratumoral or stromal infiltration were observed between hrHPV$^-$ and hrHPV$^+$ samples. In addition, to determine the subtype of macrophages infiltrating penile tumors, a fluorescent double staining of CD163 and CD68 was performed (**Figures 4A,B**) and the majority of cells were found to be CD68$^+$CD163$^+$ both intratumoral and in stromal areas, indicative of M2-polarization of virtually all macrophages in these tumors.

Univariable analyses
Associations between TME factors and LNM
Results from the univariable analysis are presented in **Table 2**. With clinicopathological parameters and updated follow-up of patients, results resembled our previous reports.[7,9,11] Tumor PD-L1 expression was significantly associated with LNM; diffusely PD-L1-positive tumors had higher odds of LNM in comparison to tumors with marginal PD-L1 expression only [OR 4.16, p < 0.01] and to tumors with combined negative/margin PD-L1 expression (OR 3.28, p < 0.01). Presence of PD-L1$^+$ TIMs was associated with higher chance of LNM but not on a level of conventional statistical significance (OR 1.91, p > 0.05).
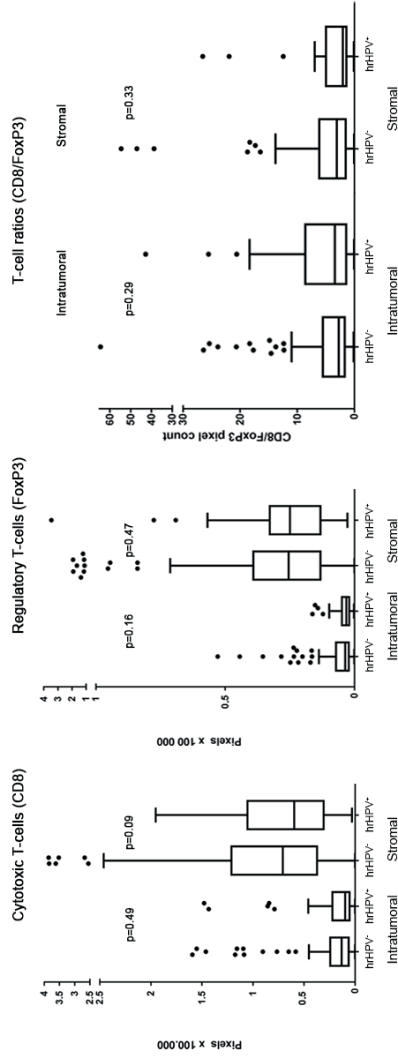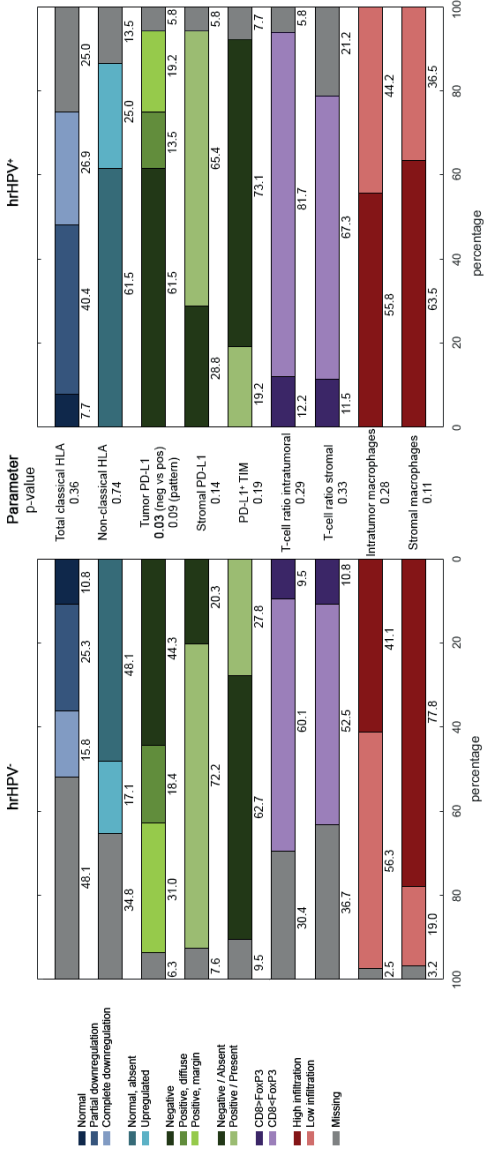
Figure 2. Tumor-microenvironmental characteristics. Plots of immune parameters in hrHPV− and hrHPV+ samples; categorical variables in bar plots, continuous variables in box plots. *p*-Values of testing comparable distribution in hrHPV groups. Table S2 in Supplementary Material presents the same data. Abbreviations: HLA, human leukocyte antigen; PD-L1, Programmed death ligand-1; TIM, tumor-infiltrating macrophages; hrHPV, high-risk human papilloma virus. Expression of PD-L1 on tumor cells was compared in two ways: negative vs. positive (neg vs. pos) and negative vs. positive at margin vs. diffusely positive (pattern).

Figure 3. Examples of representative stainings for high and low infiltration of CD8+ T cells **(A,B)**, FoxP3+ regulatory T-cells **(C,D)**, and CD163+ macrophages **(E,F)**. Scale bars: 100 µm.
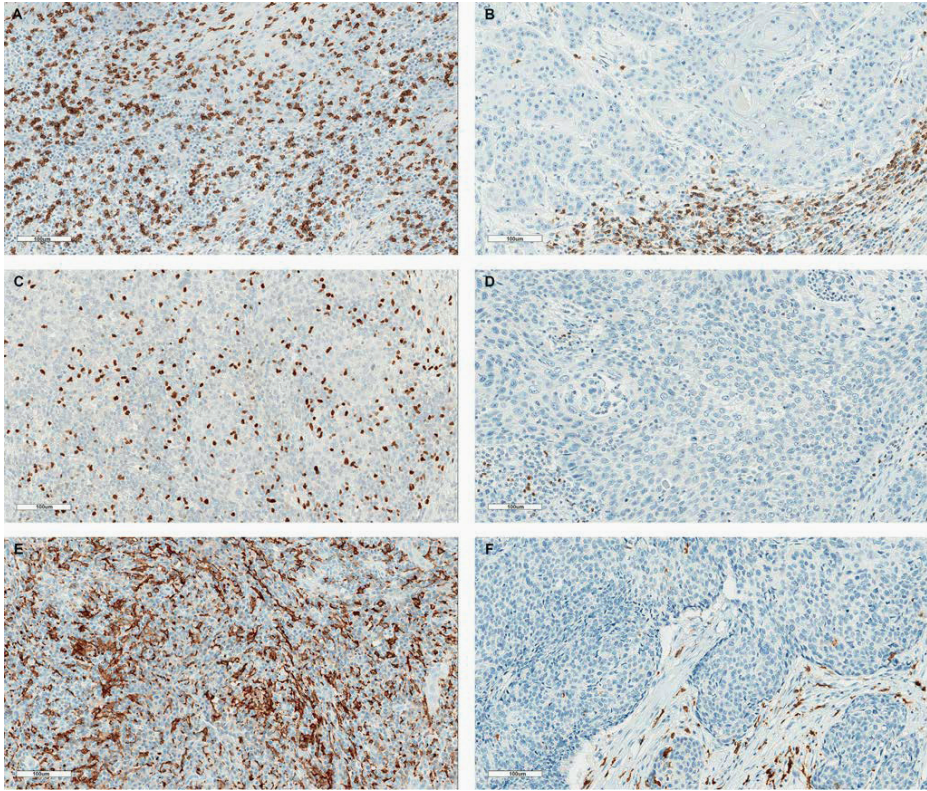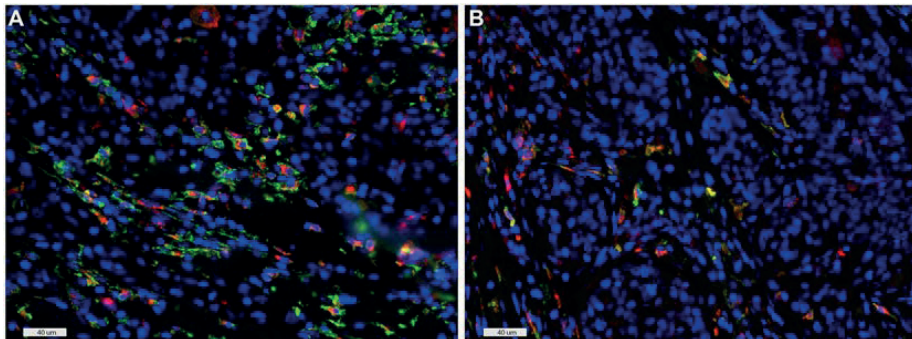

Figure 4. CD163 and CD68 double staining of an hrHPV⁻ **(A)** and hrHPV⁺ **(B)** case, indicative of M2 macrophage polarization. Colors: green, CD163; red, CD68; and blue, DAPI. Scale bars: 40 µm.

The presence of high numbers of intratumoral CD163$^+$ M2 macrophages was significantly associated with higher LNM incidence (OR 2.45, p < 0.01).

Aberrant classical HLA class I expression patterns (combined score of HLA-A, HLA-B/C, and β2m) did not show significant associations with LNM. Interestingly, upregulation of non-classical HLA class I molecules (combined score of HLA-E and HLA-G) was associated with a higher odds of LNM compared with normal expression (OR 2.28, p = 0.02).

The only T-cell infiltration parameter showing significant association with LNM, was increased CD8$^+$ T-cell infiltration rate in tumor-associated stroma (OR 0.60, p = 0.04) albeit with a confidence interval (95% CI) almost including 1: 0.37–0.98.

Associations between TME factors and DSS

High-risk human papilloma virus negativity was associated with worse survival (HR 4.82, p = 0.03), and complete downregulation of classical HLA class I with better survival than partial downregulation (HR 0.12, p < 0.05, note questionable 95% CI of 0.02–0.96) (**Table 2**). A diffuse PD-L1 tumor expression pattern was associated with higher risk of disease-specific death than marginal PD-L1 expression (HR 4.35, p < 0.01), and negative/ margin PD-L1 expression (HR 3.70, p < 0.01).

Although we saw some evidence of associations of DSS with intratumoral Tregs (HR 36.39, p = 0.06) and high intratumoral CD163$^+$ M2-macrophage infiltration (HR 2.10, p ≥ 0.05), these associations were not significant.

Multivariable analysis

Classical and non-classical HLA were non-significant in the multivariable models (data not shown). These variables limited the number of included cases in the multivariable models because of a relatively high number of missing values, and therefore they were excluded from the final models to increase the sample size.

In the multivariable analysis (**Table 3**), diffuse PD-L1 expression was the only immunological factor that remained significantly associated with LNM, although the lower limit of the confidence interval was just above 1 (OR 2.81, 95% CI [1.01–7.81], p < 0.05). hrHPV negativity and diffuse PD-L1 expression were immune factors predicting poor survival in the multivariable model (OR 9.73, p < 0.01, and OR 2.78, p = 0.03, respectively).

3

Table 2: Univariable analysis.

Variable	Contrast
Tumor-microenvironmental parameters	
High-risk HPV	Negative vs. positive
PD-L1 pattern	Negative vs. margin
	Diffuse vs. margin
	Diffuse vs. negative/margin
PD-L1 stroma	Positive vs. negative
PD-L1 TIM	Present vs. absent
Classical HLA class I	Complete vs. partial downregulation
	Normal expression vs. partial downregulation
Non-classical HLA class I	Upregulated vs. normal
CD8 intratumoral	Per 100,000 pixels
CD8 stromal	Per 100,000 pixels
FoxP3 intratumoral	Per 100,000 pixels
FoxP3 stromal	Per 100,000 pixels
T-cell ratio intratumoral	Continuous
T-cell ratio stromal	Continuous
CD163 intratumoral	High vs. low infiltration
CD163 stromal	High vs. low infiltration
Clinicopathological parameters	
Age	Per year
Tumor size	Per cm
pT stage	pT2 vs. pT1a/b
	pT3–4 vs. pT1a/b
Grade of differentiation	Intermediate vs. good differentiation
	Poor vs. good differentiation
LVI	Present vs. absent
Exophytic growth	Present vs. absent
Lymph node status	pN+ vs. pN0
Extranodal growth[a]	Present vs. absent

OR, odds ratio; HR, hazard ratio; LVI, lymphovascular invasion; HPV, human papilloma virus;
HLA, human leukocyte antigen; PD-L1, programmed death ligand-1; TIM, tumor- infiltrating macrophages.
[a]Excluding cases with no LNM.
Bold numbers are statistically significant.

	Lymph node metastasis (lnM)		Disease-specific survival (Dss)	
	Or [ci]	p-Value	hr [ci]	p-Value
	1.07 [0.55–2.11]	0.84	4.82 [1.15–20.27]	**0.03**
	1.44 [0.68–3.03]	0.34	1.28 [0.44–3.68]	0.65
	4.16 [1.71–10.17]	**<0.01**	4.35 [1.53–12.34]	**<0.01**
	3.28 [1.58–6.84]	**<0.01**	3.70 [1.75–7.82]	**<0.01**
	0.78 [0.39–1.55]	0.48	1.38 [0.52–3.63]	0.52
	1.91 [0.99–3.70]	>0.05	1.74 [0.79–3.83]	0.17
	1.26 [0.45–3.58]	0.18	0.12 [0.02–0.96]	**<0.05**
	0.69 [0.22–2.19]	0.66	0.45 [0.10–2.00]	0.29
	2.28 [1.08–4.81]	**0.02**	0.53 [0.15–1.84]	0.32
	1.32 [0.50–3.50]	0.58	0.83 [0.21–3.31]	0.79
	0.60 [0.37–0.98]	**0.04**	0.84 [0.49–1.44]	0.52
	24.74 [0.40–1,532.10]	0.13	36.39 [0.92–1,433.75]	0.06
	0.54 [0.18–1.62]	0.27	0.61 [0.14–2.57]	0.50
	1.01 [0.97–1.05]	0.71	0.96 [0.88–1.05]	0.39
	0.98 [0.92–1.04]	0.42	1.00 [0.93–1.06]	0.93
	2.45 [1.35–4.43]	**<0.01**	2.10 [0.99–4.44]	>0.05
	1.75 [0.85–3.62]	0.13	1.99 [0.69–5.74]	0.20
	1.00 [0.98–1.02]	0.95	1.01 [0.98–1.04]	0.40
	1.11 [0.97–1.26]	0.13	1.21 [1.07–1.37]	
	2.33 [1.10–4.91]	**0.03**	1.32 [0.48–3.67]	0.59
	6.57 [2.25–19.17]	**<0.01**	7.19 [2.46–21.07]	**<0.01**
	8.01 [3.16–20.27]	**<0.01**	2.11 [0.74–5.98]	0.16
	21.14 [7.32–61.11]	**<0.01**	5.99 [2.11–17.01]	**<0.01**
	4.65 [2.11–10.23]	**<0.01**	3.21 [1.45–7.10]	**<0.01**
	0.62 [0.32–1.18]	0.14	0.65 [0.28–1.53]	0.33
	-	-	38.51 [9.15–162.16]	**<0.01**
	-	-	2.11 [0.93–4.78]	0.08

Table 3: Multivariable backward regression analysis.

Lymph node metastasis			
Variable	**Contrast**	**Or [ci]**	***p*-Value**
Tumor PD-L1	Diffuse vs. negative/margin	2.81 [1.01–7.81]	**0.05**
PD-L1$^+$ TIM	Present vs. absent	–	–
CD8 stromal	Per 100,000 pixels increase	0.54 [0.27–1.05]	0.07
CD163 intratumoral	High vs. low infiltration	–	–
LVI	Present vs. absent	3.18 [1.08–9.35]	**0.04**
Grade of differentiation	Intermediate vs. gooddifferentiation	6.76 [2.11–21.63]	**<0.01**
	Poor vs. good differentiation	12.07 [3.19–45.70]	**<0.01**
pT stage	pT2 vs. pT1a/b	–	–
	pT3–4 vs. pT1a/b	–	–
Disease-specific survival			
Variable	**Contrast**	**Or [ci]**	***p*-Value**
High-risk HPV	Absent vs. present	9.73 [2.12–44.72]	**<0.01**
Tumor PD-L1	Diffuse vs. negative/margin	2.78 [1.10–6.98]	**0.03**
FoxP3 intratumoral	Per 1,000 pixels increase	-	-
CD163 intratumoral	High vs. low infiltration	-	-
Tumor size	Per cm	1.31 [1.11–1.53]	**<0.01**
Lymph node status	pN+ vs. pN0	63.21 [12.36–323.23]	**<0.01**
Grade of differentiation	Intermediate vs. good differentiation	0.30 [0.09–1.06]	0.06
	Poor vs. good differentiation	0.87 [0.26–2.90]	0.81

OR, odds ratio; CI, 95% confidence interval for OR/HR; PD-L1, programmed death ligand-1; TIM, tumor-infiltrating macrophages LVI, lymphovascular invasion; HR, hazard ratio; HPV, human papilloma virus; –, excluded during regression analysis. Bold numbers are statistically significant.

Subgroup analyses

hrHPV$^+$ and hrHPV$^-$ penile cancer can be seen as two different tumor entities, and patients with hrHPV$^-$ tumors have a higher risk of dying from this disease.[7] Also, various histological subtypes of SCC have a distinct better or poorer prognosis.[38] Therefore, analyses were repeated in the hrHPV$^-$ subgroup, and the subgroup with usual histological subtype SCC (**Tables 4** and **5**).

hrHPV-Negative Subgroup

In univariable analysis of the hrHPV$^-$ subgroup (*n* = 158), three factors were significantly associated with LNM: a diffuse PD-L1 expression pattern (OR

4.18, $p < 0.01$), high intratumoral infiltration rates of CD163+ macrophages (OR 2.17, $p = 0.03$), and— associated with lower risk of LNM—high stromal CD8+ T-cell infiltration (OR 0.45, $p = 0.02$). Diffuse PD-L1 expression pattern and high intratumoral CD163+ macrophage infiltration were also significantly associated with worse DSS (HR 6.19, $p < 0.01$ and HR 2.17, $p < 0.05$).

Multivariable regression analysis of the hrHPV− subgroup, showed grade of differentiation as the only significant factor associated with LNM (OR 15.30 and 19.34 for grades 2 and 3 compared with grade 1, both $p < 0.01$). High stromal CD8+ T cell infiltration showed some evidence of negative association with LNM but was not statistically significant (OR 0.44, $p = 0.06$). PD-L1 expression pattern was eliminated during backward selection.

For DSS in the hrHPV− subgroup, LNM (HR 82.22, $p < 0.01$) and diffuse PD-L1 expression pattern (OR 5.03, $p < 0.01$) remained the most important factors in the multivariable model. High FoxP3+ Treg infiltration rates were associated with worse DSS but did not meet statistical significance (OR 183.89, $p \geq 0.05$).

Usual histological subtype SCC
Univariable analysis in the usual histological subtypes ($n = 180$), showed—similar to the hrHPV− subgroup—significant associations with LNM for PD-L1 expression pattern (OR 3.17, $p = 0.02$) and high intratumoral CD163+ macrophage infiltration rates (OR 2.36, $p < 0.01$), and a negative association with LNM for high stromal CD8+ T-cell infiltration (OR 0.45, $p = 0.03$) (**Tables 4** and **5**). Unlike the hrHPV− subgroup, LNM were also associated with PD-L1-expressing TIMs (OR 2.10, $p = 0.04$). Poor DSS was associated with presence of diffuse PD-L1 expression pattern (HR 4.22, $p = 0.02$) and high intratumoral CD163+ macrophage infiltration (HR 2.28, $p < 0.05$).

After multivariable regression, the final model for LNM included grade of differentiation (similar values as hrHPV−), high stromal CD8 (OR 0.38, $p = 0.01$) and pT stage (OR 10.14, $p = 0.02$ for T3/T4 vs. T1). Like in the hrHPV− subgroup, PD-L1 was eliminated during backward selection. For DSS, having lymph node metastases was the most important predictor of survival (HR 124.33, $p < 0.01$). The multivariable model also included hrHPV negativity (HR 6.82, $p < 0.01$) and other clinical predictors.

DISCUSSION

This is the largest study that reports on associations of multiple TME factors with patient outcomes adjusted for clinical predictors in penile cancer.

In the total cohort, diffuse PD-L1 tumor-cell expression, CD163^{+} macrophage infiltration, non-classical HLA class I upregulation and low stromal CD8^{+} T-cell infiltration, were all associated with LNM. In the multivariable model, only PD-L1 expression remained a significant predictor for LNM (OR 2.81, p = 0.05). hrHPV negativity and diffuse PD-L1 tumor-cell expression were significantly associated with poor DSS and remained so upon correction for clinical parameters (HR 9.73, p < 0.01 and HR 2.81, p = 0.03).

The strong prognostic value for hrHPV reflects two tumor entities, similar to head-and-neck SCC and vulvar SCC.[39-41] One is hrHPV-mediated, more immunogenic, and associated with better prognosis.[41,42] The other is HPV-independent, induced by chronic irritation, inflammation and genetic alterations.[39,40,43] Interestingly, the only immune factor that differed from HPV[+] to HPV[-] tumors was PD-L1 expression, with higher PD-L1 expression rates in the latter (p = 0.03). In the HPV^{-} cohort (n=158), LNM were associated with diffuse PD-L1 tumor-cell expression, high intratumoral CD163^{+} macrophage infiltration and low number of stromal CD8^{+} T-cells, while only the first two parameters were associated with DSS. In the HPV^{-} subgroup multivariable regression model, diffuse PD-L1 expression remained significantly associated with poor DSS (HR 5.03, p < 0.01). Similar results were obtained when the cohort analysis was restricted to the usual histological subtype SCC.

The contrasting associations of diffuse PD-L1 expression with poor outcomes and PD-L1 expression at the tumor-stroma margin with more favorable outcomes can be explained by two different pathways of PD-L1 expression, identified in melanoma and gynecological SCC.[44-47] The first has a genetic background (deregulated signaling pathways, transcription factors and numerical aberrations) resulting in *CD274* overexpression, and concomitant diffuse PD-L1 expression.[15,44,46] The other is a reactive, interferon-gamma (IFNγ) induced expression at the tumor-stroma margin, explaining its favorable role.[45,47] We hypothesized that the better survival of cases with tumor-margin PD-L1 expression is explained by accumulation of activated T-cells and IFNγ release in the adjacent stroma.[11] But among the PD-L1-positive tumors, stromal CD8^{+} T-cell infiltration was not associated with a marginal expression pattern (data not shown,

Table 4: Univariable subgroup analysis.

Univariable subgroup analysis

hrHPV⁻ subgroup (N = 158)

Variable	Contrast	lymph node metastasis (LNM) OR [CI]	p-value	Disease-specific survival (DSS) HR [CI]	p-value
PD-L1 pattern	Negative vs. margin	1.67 [0.71–3.96]	0.24	1.86 [0.58–5.93]	0.30
	Diffuse vs. margin	4.18 [1.53–11.43]	**<0.01**	6.19 [2.00–19.22]	**<0.01**
	Diffuse vs. negative/margin	3.04 [1.32–7.01]	**<0.01**	4.15 [1.92–8.98]	**<0.01**
PD-L1 stroma	Positive vs. negative	0.98 [0.42–2.27]	0.95	1.42 [0.49–4.15]	0.52
PD-L1 TIM	Present vs. absent	1.52 [0.72–3.25]	0.28	1.46 [0.64–3.33]	0.37
Total classical HLA class I	Partial downregulation vs. normal expression	2.17 [0.60–7.85]	0.24	0.13 [0.02–1.01]	0.05
	Complete downregulations vs. normal expression	1.26 [0.31–5.23]	0.75	0.37 [0.08–1.66]	0.19
Total non-classical HLA class I	Upregulated vs. normal	1.84 [0.75–4.55]	0.15	0.59 [0.17–2.07]	0.41
CD8 intratumoral	Per 100,000 pixels	0.83 [0.24–2.85]	0.77	0.80 [0.18–3.62]	0.77
CD8 stromal	Per 100,000 pixels	0.45 [0.23–0.88]	**0.02**	0.80 [0.45–1.43]	0.46
FoxP3 intratumoral	Per 100,000 pixels	18.21 [0.25–1,345.66]	0.19	22.69 [0.58–891.96]	0.10
FoxP3 stromal	Per 100,000 pixels	0.71 [0.22–2.27]	0.56	0.52 [0.12–2.35]	0.40
T-cell ratio intratumoral	Continuous	1.00 [0.95–1.05]	0.96	0.93 [0.81–1.05]	0.24
T-cell ratio stromal	Continuous	0.95 [0.86–1.04]	0.24	0.99 [0.93–1.06]	0.74
CD163 intratumoral	High vs. low infiltration	2.17 [1.10–4.30]	**0.03**	2.17 [1.10–4.30]	**0.05**
CD163 stromal	High vs. low infiltration	1.43 [0.59–3.48]	0.44	1.23 [0.59–3.48]	0.44

Usual scc subgroup (N = 180)

Variable	Contrast	lymph node metastasis (LNM) OR [CI]	p-value	Disease-specific survival (DSS) HR [CI]	p-value
High-risk HPV	Negative vs. positive	1.17 [0.56–2.45]	0.68	4.04 [0.96–17.10]	0.06
PD-L1 pattern	Negative vs. margin	1.37 [0.62–3.04]	0.44	1.65 [0.52–5.17]	0.39
	Diffuse vs. margin	3.17 [1.23–8.18]	**0.02**	4.22 [1.32–13.45]	**0.02**
	Diffuse vs. negative/margin	2.58 [1.17–5.67]	**0.02**	3.01 [1.35–6.69]	**<0.01**
PD-L1 stroma	Positive vs. negative	0.79 [0.37–1.68]	0.54	1.12 [0.42–3.00]	0.82

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Table 4: Continued

PD-L1 TIM	Present vs. absent	2.10 [1.05–4.20]	**0.04**	1.70 [0.75–3.82]	0.20
Total classical HLA class I	Partial downregulation vs. normal expression	1.20 [0.41–3.52]	0.74	0.00 [0.00–0.00]	0.96
	Complete downregulations vs. normal expression	0.48 [0.13–1.75]	0.27	0.46 [0.10–2.12]	0.32
Total non-classical HLA class I	Upregulated vs. normal	2.14 [0.97–4.75]	0.06	0.59 [0.17–2.08]	0.41
CD8 intratumoral	Per 100,000 pixels	1.21 [0.38–3.80]	0.75	0.79 [0.17–3.60]	0.76
CD8 stromal	Per 100,000 pixels	0.55 [0.32–0.93]	**0.03**	0.81 [0.47–1.40]	0.45
FoxP3 intratumoral	Per 100,000 pixels	40.50 [0.53–3,085.53]	0.09	19.38 [0.34–1,114.01]	0.15
FoxP3 stromal	Per 100,000 pixels	0.0.59 [0.20–1.74]	0.34	0.69 [0.18–2.74]	0.60
T-cell ratio intratumoral	Continuous	0.98 [0.92–1.04]	0.51	0.97 [0.88–1.06]	0.45
T-cell ratio stromal	Continuous	0.96 [0.90–1.03]	0.24	0.99 [0.93–1.06]	0.92

OR, odds ratio; HR, hazard ratio; CI, 95% confidence interval for OR/HR; LVI, lymphovascular invasion; hrHPV, high-risk human papilloma virus; HLA, human leukocyte antigen; PD-L1, Programmed death ligand-1; TIM, tumor-infiltrating macrophages; SCC, squamous cell carcinoma; –, excluded from the multivariable model during regression analysis; NA, not applicable.
Bold numbers are statistically significant.

Table 5: Multivariable subgroup analysis.

Multivariable subgroup analysis

lymph node metastasis (LNM)

Variables included in model	Contrast	hrHPV−		Usual SCC	
		OR [CI]	p-value	OR [CI]	p-value
Tumor PD-L1	Diffuse vs. negative/margin	–	–	–	–
PD-L1+ TIM	Present vs. absent	–	–	–	–
CD8 stromal	Per 100,000 pixels increase	0.44 [0.18–1.05]	0.06	0.38 [0.18–0.81]	**0.01**
CD163 intratumoral	High vs. low infiltration	–	–	–	–
LVI	Present vs. absent	3.91 [0.93–18.37]	0.08	–	–
Grade of differentiation	Intermediate vs. good differentiation	15.30 [3.86–60.66]	**<0.01**	6.09 [1.82–20.44]	**<0.01**
	Poor vs. good differentiation	19.34 [3.92–95.53]	**<0.01**	19.11 [4.34–84.11]	**<0.01**
pT stage	pT2 vs. pT1	–	–	1.43 [0.45–4.48]	0.55
	pT3–4 vs. pT1	–	–	10.14 [1.39–73.84]	**0.02**

Disease-specific survival

Variables included in model	Contrast	hrHPV−		Usual SCC	
		OR [CI]	p-value	OR [CI]	p-value
High-risk HPV	Absent vs. present	NA	NA	6.82 [1.49–31.37]	**0.01**
Tumor PD-L1	Diffuse vs. negative/margin	5.03 [1.81–13.99]	**<0.01**	2.48 [0.91–6.80]	0.08
FoxP3 intratumoral	Per 1,000 pixels increase	183.89 [0.96–35,153.22]	>0.05	–	–
CD163 intratumoral	High vs. low infiltration	–	–	–	–
Tumor size	Per cm	1.47 [1.23–1.76]	**<0.01**	1.32 [1.12–1.55]	**<0.01**
Lymph node status	pN+ vs. pN0	82.22 [14.99–450.90]	**<0.01**	124.33 [14.51–1,065.27]	**<0.01**
Grade of differentiation	Intermediate vs. good differentiation	0.25 [0.07–0.91]	**0.04**	0.25 [0.07–0.92]	**0.04**
	Poor vs. good differentiation	0.84 [0.25–2.86]	0.78	0.71 [0.22–2.34]	0.57

hrHPV, high-risk human papilloma virus; OR, odds ratio; CI, 95% confidence interval; PD-L1, programmed death ligand-1; TIM, tumor-infiltrating macrophages; LVI, lymphovascular invasion; HR, hazard ratio; SCC, squamous cell carcinoma; –, excluded from the multivariable model during regression analysis. Bold numbers are statistically significant.

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Spearman, p = 0.819). The higher number of diffusely PD-L1 positive tumors in the hrHPV⁻ group of our cohort, however, fits the hypothesis of a more mutated tumor type with higher T-cell inhibition properties, partially explaining poorer survival. Deng et al. studied PD-L1 expression and tumor infiltrating lymphocytes in penile cancer and also did functional analyses on cell lines.[14] They found PD-L1 expression positively correlated with IFNγ and CD8⁺ gene expression, suggesting that indeed PD-L1 expression was induced by activated T-cells.[14,45] The proportion of hrHPV⁺ tumors in their study is presumably low (prevalence in Asia around 13%).[14] Recent studies in oropharyngeal SCC reported on a prognostic role for CD8⁺ T-cell infiltration rates and not for PD-L1 expression.[17,48] Like us, Oguejiofor et al. found higher PD-L1 expression in HPV⁻ tumors.[17] However, they also investigated CD8⁺ T-cells expressing the PD-L1 receptor PD-1 and found higher proportions of CD8⁺PD-1⁺ T-cells in stroma than in tumor. Considering higher PD-L1 expression in hrHPV⁻ tumors, this suggests pronounced T-cell inhibition in this unfavorable group. In HNSCC, CD8⁺ T-cells were more frequent in HPV⁺ tumors, and also more capable of producing IFNγ.[20] Another study found that not only composition but also location of suppressive factors matter; PD-L1⁺ or FoxP3⁺ cells close to CD8⁺ T-cells (within 30 µm) are associated with worse overall survival.[48] We did not assess PD-1 expression, IFNγ-producing capacity or proximity of suppressive factors in our cohort, but these factors may influence the different outcomes of patients with hrHPV⁺ and hrHPV⁻ tumors.

Cocks et al. found a decreased CD8⁺ T cell/FoxP3⁺ Treg-ratio associated with tumor progression during follow-up in penile cancer patients, but no associations with overall survival or DSS.[12] We also found no associations with this ratio and did not use progression during follow-up as outcome. These discrepancies can be partially explained by technical differences (they performed hot-spot analysis in TMAs). But also by factors that are not included in our analysis, such as other checkpoint molecules (e.g., CTLA-4) and PD-1 expression on T-cells.

Based on our results, can we inverse tumor escape in penile carcinomas, and how?

First, with PD-L1 as one of the most important predictors of prognosis in penile SCC, trials with PD-(L)1-checkpoint-inhibitors are warranted. Systemic treatment with these agents has been FDA-approved for various cancers, including SCCs.[49] In the Netherlands Cancer Institute, we are currently planning a clinical trial with such agents in advanced penile cancer.

Second, the favorable high stromal CD8^{+} T cell and low intratumoral CD163^{+} macrophage infiltration should be notified as important mechanisms. M2-polarized macrophages play a crucial role in T-cell response suppression, angiogenesis and treatment evasion, but can be reprogrammed towards activated M1-macrophages by CD4^{+} helper T-cells.[30,31,50] In the future, combinational immunotherapies should be applied to counter the adverse effects of the complex microenvironment in these tumors.[51]

Limitations of the study include the relatively few cases with LNM and disease associated deaths in this cohort, and the substantial missing values in HLA-expression due to insufficient tissue material for TMA sampling.[9] Both limited the statistical analysis. Second, we did not determine PD-1 expression, distance from CD8^{+} T-cells to PD-L1 expressing tumor cells and tumor-associated macrophages, or functionality.[48] Furthermore, our results ideally are externally validated.

Nevertheless, our results favor the rationale for immunotherapy for this mutilating disease. Any effectiveness of immunotherapy on primary tumor or LNM has to be revealed by future clinical studies, stratifying patients based on TME parameters, eventually leading to personalized immunotherapy. We are currently focusing on comparing the TME of primary tumors to metastatic lymph nodes.

In conclusion, in this study, we showed that the penile cancer microenvironment is highly complex and contains various targets for immunotherapy. These results can aid risk-stratification and importantly, the here described TME factors should be evaluated in future immunotherapy clinical studies to ultimately lead to patient tailored treatment.

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Supplementary Table 1: Immunohistochemical stainings.

Antibody	Clone	Catalog number	Manufacturer	Tissue sections & preparation
HLA-A	HCA2	-	Provided by prof. Neefjes (NCI, The Netherlands)	Tissue microarray. Deparaffinisation and rehydration with graded ethanol to distilled water. Endogenous peroxidase activity was blocked with 0.03% H_2O_2/ MeOH for 20'.
HLA-B/C	HC10	-		
β2 micro-globulin	A0072	anti-β-2M	DAKO, Denmark	
HLA-E	MEM-E/02	MCA2193	Bio-Rad, USA (formerly AbD Serotec, UK)	
HLA-G	4H84	557577	BD Pharmingen, USA	
CD8	C8/144B	M7103	DAKO / Agilent, Denmark	Whole mount sections. BenchMark Ultra autostainer (Ventana Medical Systems, USA). Paraffin sections were heated (28' 75°C) and deparaffinized with EZ prep solution (Ventana Medical Systems, USA).
PD-L1	E1L3N	13684	Cell Signaling Technology, USA	
FoxP3	236A/E7	vab20034	AbCam, UK	
CD163	MRQ-26	5973929001	Cell Marque, USA	
Double staining CD68/CD163	514H12 / 10D6	MCA1815 / NCL-CD163	Bio-Rad, UK / Novocastra Germany	Whole mount sections.

NCI, Netherlands Cancer Institute; RT, Room temperature; ', minutes; °C, degrees Celsius.

Heat induced antigen retrieval	Primary antibody incubation	Secondary antibody	Detection	Counterstain
10' boiling 0.01M citrate buffer at pH 6.0 for HLA-A, HLA-B/C, β2M, and HLA-E. 10' boiling Tris/EDTA buffer at pH 9.0	1:600 Overnight, RT 1:1000 Overnight, RT 1:4000 Overnight, RT 1:250 Overnight, RT 1:100 Overnight, RT	30' BrightVision (ImmunoLogic BV, The Netherlands)	10' applying a 0.05M Tris–HCl buffer containing 0.05% of 3,3'-diamino-benzidine-tetrahydrochloride and 0.0018% H_2O_2.	Mayer's haematoxylin
32' 95°C pH8.5 Cell conditioning 1 (Ventana Medical Systems, USA)	1:100 32' 37°C	OptiView DAB Detection Kit (Ventana Medical Systems, USA)		Hematoxylin II and Blueing Reagent (Ventana Medical Systems, USA)
64' 95°C pH8.5 Cell conditioning 1 (Ventana Medical Systems, USA)	1:200 60' RT 1:40 120' RT Ready to use dispenser 32' 37°C	UltraView DAB Detection kit (Ventana Medical Systems, USA)		
10' boiling Tris/EDTA buffer at pH 9.0	1:50 / 1:100 Overnight, RT	Secondary antibodies: Goat-anti-Mouse IgG2a Alexa-Fluor-594 / Goat-anti-Mouse IgG1 Alexa-Fluor-488 (both from Life Technologies, USA)	DAPI	

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Supplementary table 2: Tumour-microenvironmental characteristics of all cases and of hrHPV subgroups.

Variable	hrHPV- N=158 (%)	hrHPV+ N=52 (%)	Total N=213 (%)	p-value*
Tumour-microenvironmental characteristics				
High-risk HPV				-
Positive	-	52#	52 (24.4)	
Negative	158	-	158 (74.2)	
Missing	-	-	3 (1.4)	
Total classical HLA expression				0.36
Normal				
Partial downregulation	17# (10.8)	4 (7.7)	21 (9.9)	
Complete downregulation	40 (25.3)	21 (40.4)	62 (29.1)	
Missing	25 (15.8)	14 (26.9)	39 (18.3)	
	76 (48.1)	13 (25.0)	91 (42.7)	
Non-classical HLA score				0.74
Normal (absent)	76 (48.1)	32 (61.5)	108 (50.7)	
Upregulated	27 (17.1)	13 (25.0)	40 (18.8)	
Missing	55 (34.8)	7 (13.5)	65 (30.5)	
Tumour PD-L1				
Negative	70 (44.3)	32 (61.5)	104 (48.8)	**0.03** (np)
Positive	78 (49.4)	17 (32.7)	96 (45.1)	0.09
Diffuse	29 (18.4)	7 (13.5)		(pat)
Margin	49 (31.0)	10 (19.2)		
Missing	10 (6.3)	3 (5.8)	13 (6.1)	
Stromal PD-L1				0.14
Negative	32 (20.3)	15 (28.8)	48 (22.5)	
Positive	114 (72.2)	34 (65.4)	150 (70.4)	
Missing	12 (7.6)	3 (5.8)	15 (7.0)	
PD-L1+ TIM				0.19
Present	44 (27.8)	10 (19.2)	140 (65.7)	
Absent	99 (62.7)	38 (73.1)	54 (25.4)	
Missing	15 (9.5)	4 (4.4)	19 (8.9)	
Cytotoxic T-cells (CD8)				
Tumour (median, (IQR))	13128.8 (19980)	12209.5 (20287)	12426.4 (19544)	0.49
Stromal (median, (IQR))	70798.2 (85803)	60357.4 (80246)	66633.0 (85510)	0.09
Missing (number,(%))	36 (22.8)	2 (3.9)	38 (17.8)	
Regulatory T-cells (FoxP3)				
Tumour (median, (IQR))	3638.8 (5715.8)	3113.3250 (3913.9)	3463.3 (5250.6)	0.16
Stromal (median, (IQR))	25441.0 (26698.6)	24907.4300 (20230.7)	25293.9 (25353.4)	0.47
Missing (number, (%))	11 (7.0)	2 (3.9)	14 (6.6)	
T-cell ratio tumour				0.29
Median, (IQR)	2.7529 (4.2)	3.8101, (7.7)	3.1074 (5.5)	
CD8>FoxP3	15 (9.5)	6 (12.2)	23 (10.8)	
CD8<FoxP3	95 (60.1)	43 (81.7)	138 (64.8)	
Missing	45 (30.4)	3 (5.8)	52 (24.4)	
T-cell ratio stroma				0.33
Median, (IQR)	3.0899 (5.01)	2.2455 (3.9)	2.6186 (4.0)	
CD8>FoxP3	17 (10.8)	6 (11.5)	24 (11.3)	
CD8<FoxP3	83 (52.5)	35 (67.3)	119 (55.8)	
Missing	58 (36.7)	11 (21.2)	70 (32.8)	

				0.28
Tumour macrophages (CD163)				
High tumour infiltration				
Low tumour infiltration	65 (41.1)	29 (55.8)	95 (44.6)	
Missing	89 (56.3)	23 (44.2)	114 (53.5)	
	4 (2.5)	-	4 (1.9)	
Stromal macrophages (CD163)				0.11
High stromal infiltration				
Low stromal infiltration	123 (77.8)	33 (63.5)	157 (73.7)	
Missing	30 (19.0)	19 (36.5)	51 (23.9)	
	5 (3.2)	-	5 (2.3)	

Np, as tested negative or positive; pat, as tested over the three patterns of expression (negative, diffuse or at tumour-stroma margin); IQR, interquartile range; SCC, squamous cell carcinoma; HPV, human papilloma virus; HLA, human leukocyte antigen; PD-L1, programmed death-ligand 1; TIM, tumour infiltrating macrophages.

* Excluding missing cases. Comparing the two HPV-subgroups. Independent sample t-test for continuous variables, Chi-square or Fishers exact test for categorical variables.

One case was excluded because the majority of other variables was missing.[3,11]

Chapter 4

Advancements in staging and imaging for penile cancer

4

Sarah R. Ottenhof, Andrew R. Leone, Simon Horenblas, Philippe E. Spiess, Erik Vegt

ABSTRACT

Purpose of review: we review recent advancements in staging and imaging of penile cancer, including surveillance after therapy.

Recent findings: For cN0 patients, the sentinel lymph node biopsy (SNB) is currently considered the preferred way for invasive staging. It has largely replaced other modalities such as staging inguinal lymphadenectomy. Its diagnostic value increases when combined with other staging modalities such as ultrasound with fine needle aspiration cytology (US-FNAC) or FDG-PET/CT.

In patients with palpably suspicious nodes (cN+), imaging is more useful. PET/CT has shown good accuracy in a limited number of small studies. US-FNAC can confirm nodal metastatic disease. A staging inguinal lymphadenectomy is of therapeutic value, but is associated with significant morbidity. Omitting staging inguinal lymphadenectomy was recently described; one study showed safe use of SNB combined with intraoperative ultrasound-guided resection of suspicious nodes in cN+ patients. Therapeutic inguinal lymphadenectomy was only performed in the 42% of groins with metastases confirmed by pathology.

For M-staging, PET/CT has shown good sensitivity and its role should be further evaluated. Follow-up and surveillance rely on physical examination and US-FNAC.

Summary: Over the past decade, staging and treatment of penile cancer have become less invasive, while survival tends to improve.

1 INTRODUCTION

Penile cancer, squamous cell carcinoma in >95% of cases, is a rare (1/100,000 men in the US) yet potentially lethal malignancy which has significant geographic variations in incidence.[1] Over the past few decades the staging of penile cancer has improved patient risk stratification. Patients with extensive metastatic penile cancer still have dismal prognoses. However, in localized disease, advances in imaging and staging have enabled identification of patients at high risk of progression. This includes axial imaging with computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography with CT (PET/CT) using the radiotracer fluorodeoxyglucose (FDG), and surgical lymph node assessment techniques such as (dynamic) sentinel lymph node sampling biopsy (SNB) and modified inguinal lymphadenectomy. One study suggests that, despite a decrease in surgery for cN0 patients, their survival has improved, likely as a result of improved diagnostic modalities.[2] In this review, we present advances in staging and imaging of penile cancer with regard to clinical TNM-staging (**table 1**) and surveillance methods. Our recommendations are summarized in **table 2**.

2 T-STAGING

Penile cancer is staged clinically and pathologically according to the AJCC TNM classification.[3] Clinical T-staging of the primary lesion has traditionally relied on physical examination. Visual inspection and palpation has generally been considered sufficient to clinically stage the primary tumor.[4] Penile ultrasound has been examined in order to identify corporal infiltration. However, ultrasound has limited clinical utility especially in proximal tumors. CT has limited utility given its poor soft tissue resolution.[5,6] MRI has far superior soft tissue resolution (**figure 1,** [7]). MRI with artificial erection was described in 2007 in order to identify corporal invasion. MRI has some utility in patients suspected of having corporal invasion with inconclusive physical examination but desiring penile sparing treatments.[8–10] A recent series of 25 patients staged with MRI (no artificial erection) and physical examination found that MRI accurately predicted pathological staging in 18/25 (72%) of patients compared to 13/25 (52%) with physical examination alone**.**[11] Another study using MRI after inducing artificial erection in 100 patients found that MRI detected tunica albuginea invasion with 82% sensitivity and 74% specificity. However, for detection of urethral invasion the sensitivity and specificity were 63% and 82%.[12] Despite these advancements, physical examination remains the primary means of assessing clinical T-stage.

Table 1: TNM Classification for Penile Cancer, 7th edition, 2009(3)

TNM Clinical Classification

T – Primary Tumor

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Ta	Non-invasive verrucous carcinoma*
T1	Tumor invades subepithelial connective tissue
T1a	Tumor invades subepithelial connective tissue without lymphovascular invasion and is not poorly differentiated or undifferentiated
T1b	Tumor invades subepithelial connective tissue with lymphovascular invasion or is poorly differentiated or undifferentiated
T2	Tumor invades corpus spongiosum or cavernosum
T3	Tumor invades urethra
T4	Tumor invades other adjacent structures

*Note 1: Verrucous carcinoma not associated with destructive invasion.

N – Regional Lymph Nodes

Nx	Regional lymph nodes cannot be assessed
N0	No palpable or visibly enlarged inguinal lymph nodes
N1	Palpable mobile unilateral inguinal lymph node
N2	Palpable mobile multiple or bilateral inguinal lymph nodes
N3	Fixed inguinal nodal mass or pelvic lymphadenopathy unilateral or bilateral

M – Distant Metastasis

M0	No distant metastasis
M1	Distant metastasis

pTNM Pathological Classification

The pT categories correspond to the T categories. The pN categories are based upon biopsy, or surgical excision. Of the pM categories, only pM1 is a valid category (distant metastasis microscopically confirmed).

pNx	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis in a single inguinal lymph node
pN2	Metastasis in multiple or bilateral inguinal lymph nodes
pN3	Metastasis in pelvic lymph node(s), unilateral or bilateral or extranodal extension of regional lymph node metastasis

G – Histopathological Grading

Gx	Grade of differentiation cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3-4	Poorly differentiated/undifferentiated

Table 2: Summary of our recommendations per clinical TNM stage

Clinical stage TNM 2009	Best imaging / staging modality	Remarks
T1-4	Physical examination MRI on indication	Indications for MRI: desired penile sparing surgery with inconclusive physical examination.
N0	US guided FNAC, SNB	
N1	US guided FNAC, SNB	With repeated FNAC for suspicious lymph nodes. If pathology is negative but suspicion remains, surgical removal of the suspected node.
N2	FDG-PET/CT and US guided FNAC	If tumor-positive: ILND.
N3	FDG-PET/CT and US guided FNAC	If tumor-positive: ILND (with or without neoadjuvant chemotherapy or chemoradiation.
M0	-	
M1	FDG-PET/CT	To determine extent of metastatic disease.
Follow-up	Physical examination, US guided FNAC	CT or FDG-PET/CT may be used for confirmation of suspected recurrence / metastases.

TNM, classification of malignant tumours; MRI, magnetic resonance imaging; US, ultrasound; FNAC, fine needle aspiration cytology; SNB, sentinel lymph node biopsy; ILND, inguinal lymph node dissection; PET/CT, positron emission tomography with computed tomography; CT, computed tomography.
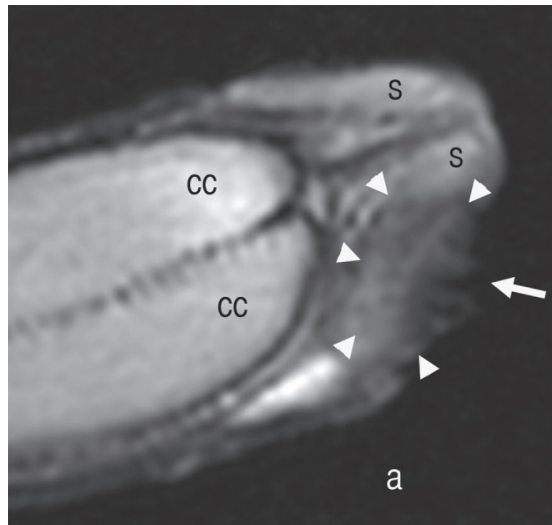
Figure 1. Magnetic resonance imaging (MRI) of penile cancer. Ulcerating lesion on the glans (white arrowheads, with a white arrow showing the ulcerated part), pT2 on histology and correctly called T2 on MRI. CC, corpus cavernosum; S, the spongiosal part of the glans. Reproduced with permission from Br J Radiol.[7]

3 N-STAGING

3.1 Introduction
Penile cancer metastasizes according to a consistent pattern: first to the inguinal lymph nodes, then sequentially to pelvic nodes, higher nodes and distant organs. Accurate lymph node staging is of the utmost importance to determine the optimal treatment. The prognosis of patients without lymph node metastases is excellent (cure in >80%), but decreases progressively with higher stages of lymph node involvement.[2,4]

3.2 Physical examination
At physical examination, the groins are palpated for suspicious lymph nodes, their number, size, and mobility. Physical examination is not very accurate, especially in detecting small metastases, but grossly identifies two risk groups: those with palpable nodes (cN+) and those without (cN0). The majority of new patients present without palpable nodes, but 10-25% of them have occult metastases.[13–15] Of patients with palpable lymph nodes in the groins, the majority have metastatic disease. Fixed inguinal nodes are almost always metastatic.[4] If physical examination is complicated, e.g. by obesity, imaging can be used.

3.3 Prediction of lymph node metastases
Lymphovascular invasion (LVI) at the primary tumor site is one of the strongest predictors of occult lymph node metastases.[16,17] Other predictive factors are perineural invasion, grade of differentiation, superoxide dismutase-2 expression, tumor depth, and clear cell differentiation.[18–20] Several authors developed nomograms to predict lymph node involvement using aforementioned factors, physical examination and other factors. Nomograms varied from complex, including detailed pathological features,[21] to more simplified[17,22] with concordances (the proportion of correctly predicted outcomes) between 88% and 74%. A nomogram composed of primary tumor features only, resulted in an index-score that significantly correlated with lymph node status (p<0.01).[23] Another nomogram, tested in cN0 patients only, showed a concordance of 79%.[24] None of these nomograms have been externally validated.

Prediction is not accurate enough for reliable clinical N-staging. Intermediate and high risk tumors (pT1 grade 2 and worse) require further clinical and/or pathological staging. Lymph node metastases of the pelvis are best predicted by pathological characteristics of the inguinal lymph node dissection (ILND) specimen.[25]

3.4 Further staging of non-palpable lymph nodes
Conventional Imaging
Few studies have evaluated computed tomography (CT) for non-palpable inguinal node staging. In the most recent study, CT detected all 4 inguinal metastases in 44 cN0 groins.[26] However, CT mainly detects lymph node metastases based on size, and therefore is not sensitive for small metastases, despite improving spatial resolution and CT technology.

MRI is considered the most sensitive for lymph node staging, because of its better soft tissue resolution and identification of intranodal features.[27] Features such as necrosis are very specific for lymph node involvement, but certainly not all metastases show such features. A recent study compared preoperative MRI with pathologic evaluation after LND. MRI correctly staged 13 out of 15 patients (86.7%), with the remaining two cases false-positively classified as N1. Although there were no false-negatives in this per-patient analysis, a missed case of inguinal lymph node involvement is mentioned in the manuscript.[11] A relatively new technique for imaging lymph node metastases is lymphotropic nanoparticle-enhanced MRI. It has shown favorable results in prostate cancer.[28] One study has shown promising results in seven penis cancer patients, but requires further validation.[29]

Conventional imaging is associated with false-positive detection of enlarged reactive lymph nodes, and failure to detect micrometastases in normal-sized lymph nodes. Therefore, MRI and CT are of limited value for inguinal lymph node staging, but do aid staging when physical examination is difficult.[4,10]

PET/CT Imaging
A high FDG-uptake in most primary penile tumors and lymph node metastases suggests that FDG PET/CT is suitable for penile cancer staging.[30] However, PET/CT is also hampered by low sensitivities for smaller metastases. In initially cN0 staged patients, detection of inguinal involvement using FDG PET/CT established sensitivities from 20% to 80%.[26,30,31] In a meta-analysis, the pooled sensitivity in cN0 patients was only 57%.[32] Therefore, FDG-PET/CT is not recommended for cN0 patients.

Ultrasound and Fine Needle Aspiration Cytology
Ultrasonography (US) of the groins is a noninvasive, inexpensive and quick method to detect suspicious nodes. Lymph nodes are well assessable and US-guided fine needle aspiration cytology (FNAC) can prove metastatic lymph nodes

in an outpatient setting.[33] In a study assessing 83 cN0 groins, 34 groins were suspicious on US and subsequently underwent aspiration cytology. Nine groins contained malignant cells, resulting in immediate (i.e. first operation) ILND in 9 of 23 positive groin regions (sensitivity 39%, specificity 100%). In the other 14, nodal metastases first had to be proven by SNB before ILND was performed in a second operation.[34]

Because of its low sensitivity, staging with US-FNAC should be combined with more invasive staging, such as SNB, in FNAC-negative cases. Combined procedures will be discussed in the sentinel node biopsy section. FNAC is recommended in both cN0 and cN+ groins.[35]

Sentinel Node Biopsy

In penile cancer the sentinel node is always located in the inguinal region, and is typically found by injecting a blue dye and a radioactive tracer in/around the primary tumor.[36-38] Preoperative localization of the sentinel nodes by scintigraphy helps intraoperative navigation. In addition to static lymphoscintigraphic images, dynamic images may show the route of drainage to the lymph nodes in a so-called dynamic sentinel node biopsy (DSNB) procedure.[39] A study (n=280) showed slightly but insignificantly superior results for lymphoscintigraphy on the same day (n=65 groins) compared to the day before SNB (n=215 groins).[40] Besides removal of the sentinel nodes, the procedure also entails a thorough intraoperative palpation of the groin area and excision of any suspicious lymph nodes.

As an alternative to traditional radioactive tracers combined with blue dye, recently the use of an investigational hybrid tracer was introduced, which is both radioactive (^{99m}Tc-nanocolloid) and fluorescent (indocyanine green), and follows the same drainage patterns as radioactive-only tracer.[41,42] This hybrid tracer can aid intraoperative navigation to sentinel nodes, by enabling visual detection of the radioactive nodes (**figure 2**).[43] Its detection rate appears to be higher than the detection rate of blue dye alone.[41,44]

The contemporary sentinel node procedure is a reliable staging tool with minimal morbidity and acceptable sensitivity.[45-47] In a two center analysis the false-negative rate (FNR) was 7%, with corresponding 93% sensitivity.[48] Nevertheless, for every (false) negative procedure, the patient's prognosis decreases greatly when metastases become apparent. False negatives in SNB may be caused by macroscopic metastases blocking influx into the actual sentinel node(s).[49]
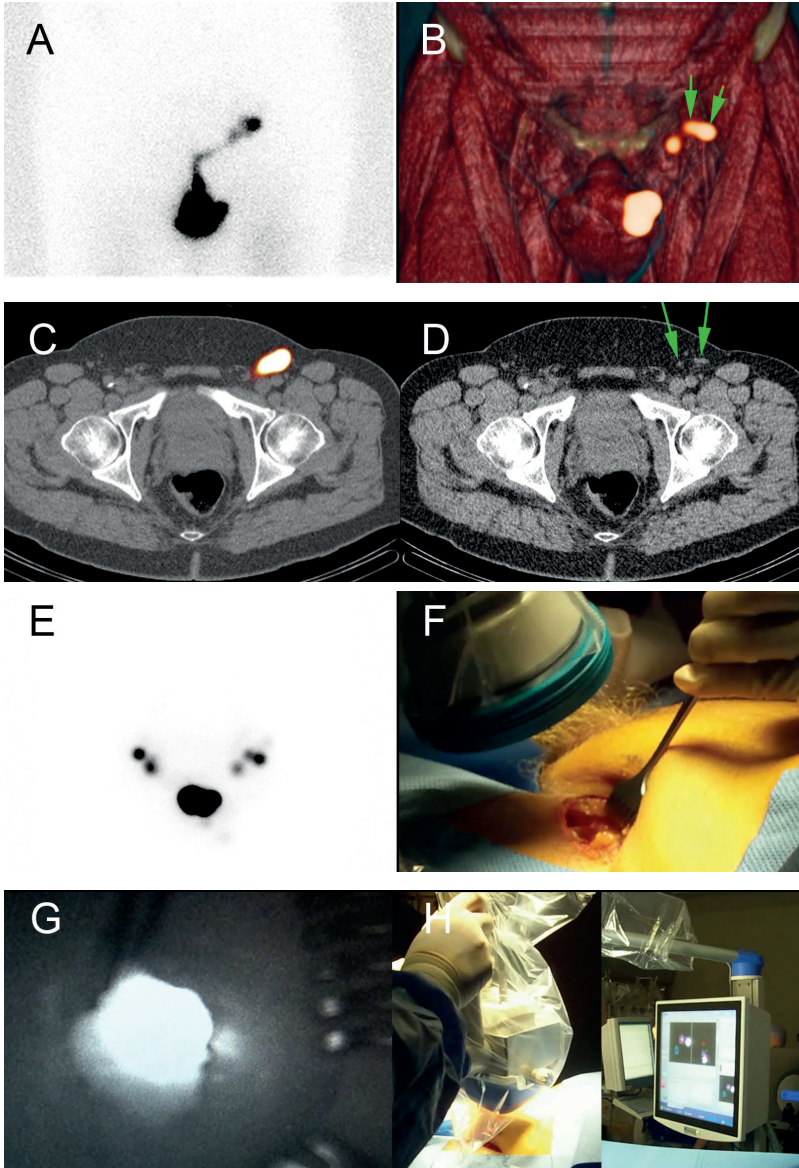
Figure 2. Dynamic sentinel node biopsy procedure of penile cancer using a hybrid radioactive-fluorescent tracer (^{99m}Tc-nanocolloid-ICG). Planar scintigraphic images 10 min after injection of radiotracer in four deposits around the penis (A) show lymph vessels from the tumor to left inguinal sentinel nodes (SNs). Volume rendered (B) and transversal (C and D) SPECT/CT images show the exact location of the SNs preoperatively. Because of right-sided non-visualization, a reinjection was performed. Scintigraphy at 1 to 2 h after reinjection (E) also shows SNs in the right groin. During surgery, a fluorescence camera (F) enables easier identification of fluorescent SNs (G). A mobile gamma camera (H) can be used to check whether all radioactive SNs are successfully removed.

Combining the (D)SNB with other staging tools that detect macroscopic metastases may reduce the FNR. Various studies used a combination of preoperative US with or without FNAC and (D)SNB.[35,47,50,51] The FNR was a very acceptable 5%; lower than the FNR of DSNB alone.[47]

A Danish study (N=129 patients) combined FDG-PET/CT imaging with SNB.[13] The combined procedure showed a sensitivity over 94% (FNR 5.6%).[13] This supports the idea that the different modalities should be used in a complimentary fashion.

Modified Inguinal Lymphadenectomy

A modified inguinal lymphadenectomy (MIL) entails the surgical removal of all the lymph nodes containing first-line lymphatic invasion. One of the problems associated with MIL is that no consensus exists on the boundaries of the dissection. Two studies have suggested that the proposed boundaries should be extended.[37,52] Unfortunately, both studies used different anatomical fields, and the optimal boundaries of MIL remain to be defined. Furthermore, its oncological safety has been questioned, although its false-negative rate seems comparable to that of SNB.[53] Nowadays, the MIL is largely replaced by the SNB procedure, but it can be used as an alternative if identification of a sentinel node fails.[54]

3.5 Further staging of palpable nodes (cN+)
Conventional Imaging

CT and MRI can detect pelvic and retroperitoneal nodes that cannot be detected by physical examination. However, their sensitivity is limited, especially for small metastases, since increased nodal size is the main criterion for malignancy. Additional criteria, such as central necrosis and irregularity of the lymph node border on CT performed well in preoperative risk stratification.[55] Nevertheless CT has a sensitivity of only 20 - 38% for the detection of pelvic lymph nodes.[25,55] Risk stratification by inguinal lymph node characteristics such as number and extranodal extension on pathologic evaluation was more reliable.[25] In an aforementioned study, MRI correctly staged all N3 patients, but this concerned only 3 patients.[11]

In a meta-analysis comparing CT and MRI of pelvic nodes in prostate cancer patients, pooled results were comparable, although the results of the individual studies varied widely. The pooled sensitivity was 42% for CT and 39% for MRI and pooled specificity was 82% for both.[56]

FDG-PET/CT

In a meta-analysis, detection of inguinal metastases with FDG-PET/CT in clinically suspected groins had a pooled sensitivity and specificity of 96 and 100% respectively.[32] Only one study evaluated FDG-PET/CT detection of pelvic nodes in penis cancer. With pathologically proven inguinal lymph node metastases, its sensitivity was 91% and its specificity was 100%.[57] PET/CT is likely to be more accurate than CT alone in the preoperative staging of the pelvic lymph nodes, as shown in other malignancies.[58-60]

Ultrasound and fine needle aspiration cytology

In clinically node-positive patients, FNAC of suspicious nodes can confirm metastases with a higher sensitivity than in cN0 patients. A study with 16 cN+ patients showed a sensitivity of 93% and a specificity of 91% of FNAC without US.[61] Another series of 28 patients showed sensitivity and specificity of both 100% in cN+ groins.[62] Nevertheless, a risk of false-negatives exists and negative aspiration biopsies in cN+ patients should be repeated.

Sentinel node biopsy

As a sentinel node biopsy also includes excision of clinically suspicious nodes, this could be performed in patients where FNAC fails to prove metastases but clinical suspicion remains. However, in those cases an SNB is less reliable, as rerouting of tracer due to tumorous obstruction is likely.[49] Therefore, repeated FNAC and excision biopsy are indicated for palpable lymph nodes. A recent study (n=36 groins) combined the sentinel node procedure with intraoperative US examination and surgical removal of suspicious lymph nodes. Therapeutic inguinal lymphadenectomy was only performed if metastases were confirmed by pathology, and could be omitted in 58% of groins. No relapses occurred during a median follow-up of 42 (range 16-84) months, yielding a false-negative rate of 0% (sensitivity 100%).[51]

Inguinal lymphadenectomy

Traditionally, an inguinal lymphadenectomy was performed for staging, but this is associated with significant complications.[63,64] Its therapeutic value is advantageous, especially in highly suspicious cases. However, cases with fixed inguinal nodes (clinically N3) require multimodal treatment such as neoadjuvant chemotherapy prior to surgery.[65]

4 M-STAGING

4.1 Introduction
In patients with inguinal nodal disease, M-staging is indicated. Considering the poor prognosis of patients with distant metastases, M-staging is mainly important to prevent futile invasive treatments.

4.2 Conventional imaging
Pelvic, abdominal and thoracic imaging is recommended with CT or MRI scanning. A chest X-ray is considered sufficient for thoracic imaging, but CT is considered more sensitive. PET/CT can be made as an alternative.[4]

4.3 FDG-PET/CT
Various distant metastases of penile cancer detected by FDG-PET/CT have been described in literature.[66–68]

In a recent study among 48 stage 3 and 4 patients, FDG-PET/CT was compared to conventional imaging (validation by both follow-up scans and biopsy) and showed a total sensitivity of 82% and specificity of 93%. In biopsy-proven lesions only, the sensitivity was 83%.[69] However, selection bias and limited follow-up in this study should be taken into account with interpretation. The exact role of PET/CT for metastases detection needs further evaluation.[70]

5 SURVEILLANCE, RESPONSE EVALUATION AND FOLLOW UP

Surveillance of all penile cancer patients relies upon physician and patient examination. Local recurrence is more common after penile sparing surgery than after partial or total penectomy, and occurs in up to 28% of patients.[71] US-FNAC for suspicious findings may improve diagnosis of regional recurrences.[33,34,72] In patients with pathologically positive nodes (pN+) at initial treatment, regular physical examination with optional imaging (CT/MRI) and US-FNAC is recommended according to NCCN and EAU guidelines.[4,65] In N2/N3 patients, NCCN guidelines recommend routine imaging of chest, abdomen and pelvis. There have been few recent advances in surveillance of penile cancer patients. PET/CT is generally reserved for confirmation of possible recurrence and is not recommended for routine surveillance.

6 CONCLUSIONS

T-staging relies on physical examination, but MRI can aid a more sensitive staging if needed.

N-staging is most complicated for small metastases, as they are prone to be missed by physical examination, sectional imaging modalities, and FNAC. Larger metastases are more likely to be detected by all aforementioned methods. For the detection of small metastases, the (dynamic) sentinel node biopsy procedure is preferred because it provides accurate pathological staging without the side effects and complications of an inguinal lymph node dissection. SNB should be preceded by US-guided FNAC to avoid undetected macrometastases. FNAC also is a valuable tool to confirm metastases in suspicious groins. Nevertheless, micrometastases can be missed with every non-invasive or minimally invasive staging tool. Additional efforts should be made to further reduce false-negative rates.

For M-staging, conventional anatomical imaging is recommended according to guidelines. High sensitivity has been described for FDG-PET/CT, but its role needs further evaluation.

Follow-up and surveillance rely on physical examination and US-FNAC. PET/CT may have a role in localization of suspected recurrence, but is not recommended for routine surveillance.

Key points
- T-staging of penile cancer relies mainly on physical examination, but MRI can provide a more accurate staging if needed.
- The diagnostic value of the sentinel node biopsy procedure increases when combined with other diagnostic procedures such as US-FNAC, PET/CT or intraoperative ultrasound examination.
- FDG-PET/CT has shown good accuracy in both clinically suspicious groins and in detecting distant metastases, but its role needs further evaluation.
- Follow-up and surveillance rely primarily on physical examination and US-FNAC.

Special and outstanding references

(11) Confirms an additional value of MRI, without artificial erection.

(13) This study shows improvement of the sentinel lymph node biopsy (SNB) by adding FDG-PET/CT, improving sensitivity to 94%. As the prognosis drops sharply after a false-negative SNB, efforts should be made to further improve sensitivity.

(51) This study presents the feasibility of minimally invasive staging in cN+ patients. They performed SNB in patients with clinically suspicious groins, and combined it with intraoperative US-guided removal of suspicious lymph nodes. Traditionally there is no role for SNB in clinically suspicious groins, but the results from this study suggest that minimally invasive staging is also safe in a selected group of cN+ patients.

(14) This study from the National Penile Cancer Register provides a nice overview of incidence and patient characteristics and tumor properties. They also provide detailed information on their staging procedures and the prognosis per clinical stage.

(69) This study evaluates FDG-PET/CT performance for staging or re-staging of penile cancer. Detailed information on accuracy for detection of metastases is given. Unfortunately, figures for lymph node staging are not specified separately for inguinal and pelvic lymph nodes.

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Chapter 5

The role of PET/CT imaging in penile cancer

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Translational Andrology and Urology

ABSTRACT

Positron emission tomography (PET) imaging with ^{18}F-fluorodeoxyglucose (FDG) combined with computed tomography (CT) provides functional imaging combined with anatomic information, improving diagnostic accuracy and confidence.

Although virtually all primary penile tumors are FDG-avid, PET/CT is not recommended for primary tumor staging as it has limited spatial resolution and is hampered by urinary FDG excretion.

The accuracy of PET/CT for lymph node staging seems to improve with the pretest likelihood of metastatic nodes. In groins with normal physical examination, sensitivity is only 57%. In groins with palpably enlarged lymph nodes, sensitivity of PET/CT reaches 96%. For pelvic lymph nodes and distant metastases, PET/CT is more accurate if inguinal metastases are present. However, these results are based on a very limited number of studies.

Overall, the role of PET/CT imaging in penile cancer remains ambiguous, especially in inguinal lymph nodes. During staging and follow-up, it may be particularly useful in detecting pelvic lymph node metastases and occult distant metastates prior to systemic chemotherapy and/or extensive surgery, insuring a better selection of patients that are most likely to benefit from such therapies.

1 BACKGROUND

Cancer of the penis is a rare disease with an incidence of 0.6-2.1 in Western countries.[1-3] The majority of these cancers are squamous cell carcinoma, but other histological types and subtypes such as basaloid and warty squamous cell, and melanoma can occur. The worldwide incidence has significant geographic variations, which is mainly explained by circumcision, sexual practices and socioeconomic circumstances.[2]

Imaging and other staging techniques have improved the risk stratification of penile cancer. Ultrasound-guided fine needle aspiration biopsy, (dynamic) sentinel node biopsy, and various forms of lymph node dissections are invasive techniques that have improved lymph node staging. In addition, axial imaging such as CT, MRI and PET/CT have also evolved in the past decades.

Positron emission tomography (PET) imaging with ^{18}F-fluorodeoxyglucose (FDG) is based on cellular uptake of glucose and FDG, which is elevated in malignant cells and other tissues with an elevated glycolytic rate.[4] FDG-PET combined with computed tomography (CT) provides functional imaging combined with anatomic information, improving diagnostic accuracy and confidence. PET/CT scanners stack PET and CT images in a single scanner, and are nowadays widely used.

In various malignant diseases, PET/CT is used for staging and monitoring. Combining functional with anatomical imaging, PET/CT outperforms both PET alone and CT alone in lymph node staging of many types of tumors.[5-7] Here we review the role of PET/CT in the diagnosis and management of penile cancer.

In penile cancers, both the primary tumors and lymph node metastases generally show high uptake of FDG. Hence, suitability for staging penile cancer is likely.[8] However, sensitivity of PET/CT is limited by its spatial resolution, reducing sensitivity for small metastases. In addition, false-positives may occur due to inflammation, such as in reactive lymph nodes.

2 PRIMARY TUMOR STAGING

In their first study on PET in penile cancer, Scher et al. found that only 6 out of 8 primary tumors were detected by FDG-PET/CT[8], but this can be explained by the small size of the two undetected tumors, which was below the spatial resolution

of PET (one residual lesion after incomplete resection of a T1 tumor, and one very small (<0.5cm) T1 tumor. The authors even describe that in retrospect both lesions did show some FDG-uptake.

In our experience, almost all primary penile tumors are FDG-avid. An example is shown in **Figure 1**. However, PET/CT is not very useful for primary tumor staging, because its limited spatial resolution makes it unsuitable for evaluating ingrowth in surrounding structures or even the exact size of the tumor. Additionally, FDG is excreted via the urine, which can interfere with imaging of the primary tumor. Physical examination and, in selected cases MRI, have excellent accuracy for staging primary penile tumors.[9]
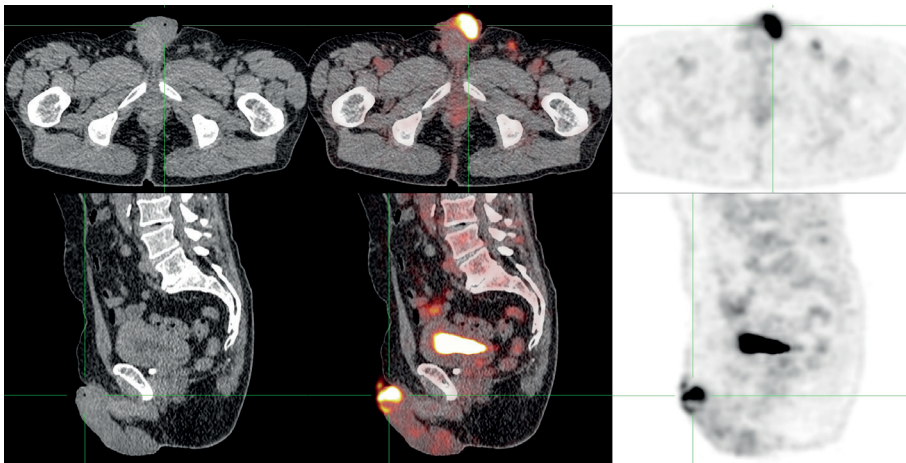
Figure 1. FDG-PET/CT of a penile cancer patient. The primary tumor exhibits high FDG uptake (SUV_{max} 11.7).

3 LYMPH NODE STAGING

The diagnostic value of FDG-PET/CT for lymph node staging varies in different types of cancer. Often, reported sensitivities are higher than for conventional modalities such as CT and MRI, but are not always satisfactory.[4] In pancreatic cancer for example, PET/CT shows a sensitivity in N-staging of only 30%.[10] Somewhat better results appear in clinically lymph node negative non-small cell lung cancer (stage I) and head and neck cancer (cN0) patients, with sensitivities for regional lymph nodes of 57% and 67% respectively.[11,12] Specificities may be higher, but identification of patients where invasive staging or therapy can be omitted requires high sensitivity.

3.1 Inguinal lymph nodes

Non-palpable lymph nodes

Detection of inguinal involvement using FDG-PET/CT in penile cancer patients that were initially staged as node negative (cN0) was evaluated in several studies.

In 2005, Scher et al. examined thirteen mostly cN0 patients. Of 16 positive lymph nodes, FDG PET/CT correctly identified 15 as positive, with only one false negative lesion, resulting in a sensitivity per lesion of 94%. The sensitivity for superficial inguinal lymph nodes was 89% per lesion and 80% per patient.[8]

Later, Leijte et al. published PET/CT results of solely cN0 groins. Five out of 42 groins had lymph node metastases, but PET/CT only identified one, resulting in a sensitivity of only 20%. The true positive lymph node metastasis was larger than the other four (30 mm vs. 10, 10, 1 and 1 mm). This study emphasizes the limited sensitivity of PET/CT in detecting small metastases.[13]

In 2012, Souillac et al. showed better sensitivity for PET/CT in cN0 patients, albeit in a small set of patients. PET/CT correctly identified 3 out of 4 metastases (75% sensitivity).[14]

In all these three studies, specificity of PET/CT was higher than sensitivity: Scher et al. 100%, Leijte et al. 92% and Souillac et al. 88%. Negative predictive values (NPV) were 89%, 90% and 83% respectively.

In a meta-analysis of seven studies by Sadeghi et al., the pooled sensitivity per groin for FDG-PET/CT in cN0 patients was only 57%.[15] Positive predictive values were also disappointing (between 25 and 37 percent). Therefore, surgical staging is necessary to identify small inguinal lymph node metastases and FDG-PET/CT is not recommended for staging of cN0 patients.[8,13–15]

Palpable lymph nodes

For patients that present with palpable lymph nodes in the groin, PET/CT has a different clinical value (**Figure 2**). The aforementioned meta-analysis by Sadeghi et al. found that detection of inguinal metastases with FDG-PET/CT in clinically suspicious groins had a pooled sensitivity and specificity of 96 and 100% per groin respectively.[15] Thus, PET/CT is more accurate in patients with palpable lymph nodes. Assessing the number of lymph nodes involved with PET/CT may be useful when neoadjuvant chemotherapy will be considered in case of multiple or bulky inguinal metastases.[16]

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3.2 Pelvic lymph nodes

On pelvic lymph nodes, literature is scarce. In a study on 18 patients with proven inguinal metastases, the diagnostic accuracy of FDG-PET/CT was evaluated for staging pelvic lymph nodes.[17] The clinical benchmark this was compared to was either histopathology, clinical follow-up, or radiologic imaging. Twenty-eight of 36 pelvic basins were eligible for analysis, and results were good. Analysis showed a sensitivity of 91%, specificity of 100%, and similar negative and positive predictive values (94 and 100 percent respectively). FDG-PET/CT gave a correct prediction in 96%. This is the only study that provides numbers on pelvic lymph node evaluation of PET/CT.

In stage 3 and 4 patients, Zhang et al. showed good results for lymph nodes (all locations) with a sensitivity of 93% and a specificity of 85%.[18] Also in other malignancies and lymph node areas, results suggest that FDG-PET/CT has higher accuracy than CT only in staging pelvic lymph nodes.[5,6,19,20] However, results are not always optimistic. A prospective multicenter trial in advanced cervical cancer found no significant improvement of sensitivity nor specificity for detection of pelvic lymph node metastases with adding FDG-PET/CT to diagnostic contrast enhanced CT.[21] In addition, Lin et al. found lower diagnostic accuracy for PET/CT than CT or MRI in a per scan receiver operating characteristic (ROC) curve analysis for metastatic pelvic lymph nodes or distant metastases in vulvar carcinoma.[22]

4 DISTANT METASTASES

Literature on distant penile cancer metastases detected with FDG-PET/CT consists largely of case reports.[23-26] In the aforementioned study by Graafland et al. among 18 patients with pathologically confirmed inguinal lymph node metastases, five patients were diagnosed with distant metastases by PET/CT.[17] Four of these five metastases were confirmed with either CT alone or histopathology.[17] Although this was a small number of patients, these results suggest that PET/CT is useful for accurate M-staging.

The largest series by Zhang et al. (2016) compared FDG-PET/CT to conventional CT in 42 patients with suspicious lesions on CT or MRI, leading to an implicit selection bias.[18] With histopathology or follow-up imaging as a reference, sensitivity and specificity of FDG-PET/CT were 85% and 86% respectively for all metastatic sites, including lymph nodes, lung, bone, etc. In a patient-based

analysis this was 82% and 93% respectively. Drawbacks of this study are a short follow-up of patients as well as the aforementioned selection bias.
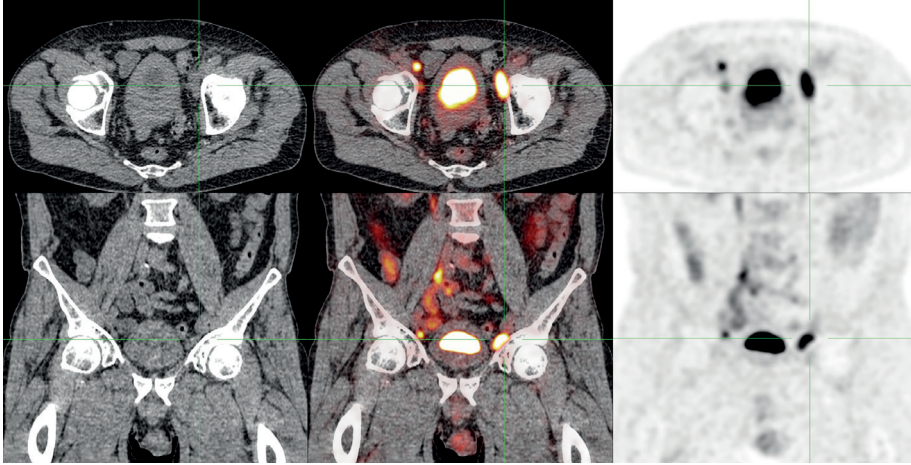

Figure 2. FDG-PET/CT of the same penile cancer patient, who was clinically staged as positive for inguinal lymph node metastases (cN+). The PET shows extensive pelvic lymph node metastases. Bilateral inguinal metastases were also FDG-positive.
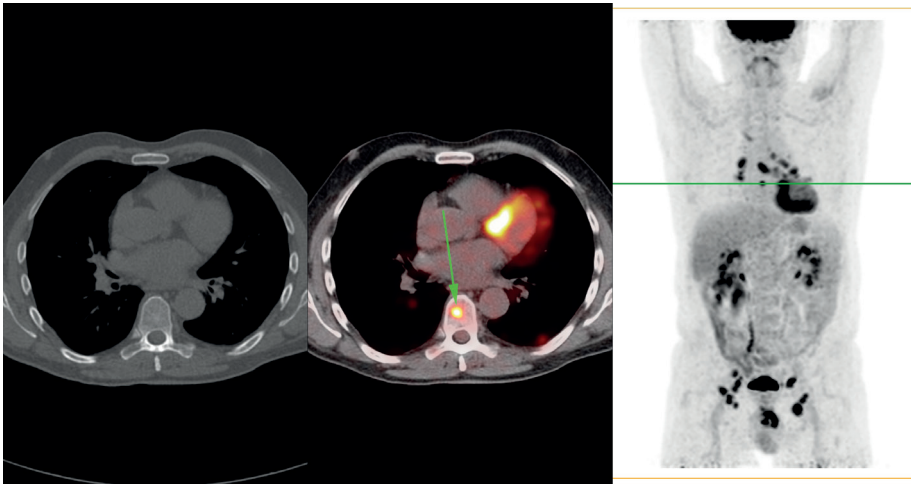

Figure 3. FDG-PET/CT of the same penile cancer patient, showing a solitary skeletal metastasis in the 8th thoracic vertebra, which was not visible on CT. In addition, extensive mediastinal and hilar lymphadenopathy was visible with small pulmonary and pleural lesions, which were thought to be possible sarcoidosis or metastases. Follow-up CT after 3 months showed gross progression, with multiple metastases in bone, liver, spleen and pelvic lymph nodes. In contrast, the lesions in the lungs and mediastinum were stable, increasing the likelihood of those being caused by a separate process such as sarcoidosis.

The whole body imaging aspect of PET/CT scanning can be of crucial impact on clinical decision making. If distant metastases are found in patients scheduled for locoregional therapy (**Figure 3**), this changes their indicated treatment to systemic or palliative regimens.[18]

5 OTHER SETTINGS

5.1 Surveillance
In general, surveillance after treatment of penile cancer depends on physical examination by the physician and the patient himself. In our experience, PET/CT can be of value for identification of the location and extent of suspected recurrence. Routine surveillance with PET/CT is not recommended.

5.2 Combined modalities
A recent study with 129 patients used FDG-PET/CT scanning complementary to sentinel node biopsy.[27] In this cohort, FDG-PET/CT found one additional metastatic lymph node that was not detected with the radiotracer of the sentinel node biopsy procedure. The combined procedure had a sensitivity of 94%, reducing the false negative rate to an acceptable level.

6 CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, the exact role of FDG-PET/CT imaging in penile cancer remains ambiguous. So far, PET/CT has shown to have significant limitations, especially in inguinal lymph node staging. Results seem to improve with the pre-test likelihood of metastatic involvement.

PET/CT may be particularly useful in assessing pelvic metastases and occult distant metastatic burden prior to systemic chemotherapy and/or prior to an extensive surgical resection. This would improve the selection of those who are most likely to benefit from an aggressive multimodal approach. However, evaluation of this indication for FDG-PET/CT in larger cohorts is warranted.[28]

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Chapter 6

F-18 Fluorodeoxyglucose positron emission tomography with computed tomography has high diagnostic value for pelvic and distant staging in patients with high-risk penile carcinoma

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ABSTRACT

Background: For penile cancer patients with pelvic metastases multimodal treatment is advised, but pelvic lymph node metastases are often found upon surgical resection only. Early selection for multimodal treatment requires reliable non-invasive staging.

Objective: To evaluate the diagnostic value of 18F-fluorodeoxyglucose positron emission tomography with computed tomography (FDG-PET/CT) for staging pelvic lymph nodes and distant metastases in high-risk penile cancer patients.

Design, setting and participants: FDG-PET/CT scans performed in patients with clinically overt inguinal lymph node metastases and/or high-risk primary tumors (bulky T3 or T4) were retrospectively analyzed.

Outcome Measurements and Statistical analysis: All scans were reviewed by two independent nuclear medicine physicians staging the pelvic nodes and distant metastases. FDG-PET/CT findings were compared to histology after node dissection if available, or to positive imaging or follow-up of at least 1 year.

Results and limitations: Between 2006 and 2016, 61 patients met the inclusion criteria. For staging of pelvic nodes, sensitivity was 85% (specificity 75%, negative predictive value (NPV) 90%, positive predictive value (PPV) 65%). For the detection of distant metastases FDG-PET/CT had a PPV of 93%. Results are limited by the retrospective design and the lack of direct comparison to CT scanning alone.

Conclusions: FDG-PET/CT has a high sensitivity and NPV for staging of pelvic lymph nodes in high-risk penile cancer. FDG-PET/CT also has high PPV for detection of distant metastases, which were found in 23% of patients. Therefore, FDG-PET/CT enables early selection for multimodal treatment of patients with pelvic metastases, and may help avoid futile treatment of patients with distant metastases.

Patient summary: We studied whether PET/CT-scans in patients with advanced penile cancer can detect metastases before lymph node surgery is done. PET/CT-scans can detect or rule out pelvic lymph node metastases and can detect distant metastases. This helps making timely treatment decisions (before surgery).

INTRODUCTION

Penile cancer is a rare disease with an incidence of 1.3/100.000 in Western countries.[1] It invariably metastasizes first to the inguinal lymph nodes on one or both sides, and then to the ipsilateral iliac lymph nodes. Patients with early stage disease have a good prognosis, but this decreases rapidly with the extent of lymph node involvement.[2-4]

Patients diagnosed with clinically overt pelvic lymph node metastases do not fare well with surgery alone.[5,6] Multimodality treatment is considered essential. The most propagated scenario so far is neoadjuvant chemotherapy followed by surgery. Whether chemoradiation with or without surgery is a viable option is not clear at this moment.[7,8] Pelvic node involvement is often found or confirmed after surgery only. In order to select these patients at the outset for neoadjuvant chemotherapy or chemoradiation, reliable non-invasive staging of the pelvic nodes would be very helpful.

Unfortunately, early detection of pelvic nodal involvement with computed tomography (CT) and/or magnetic resonance imaging (MRI) is not very accurate. Both have a high specificity (60-100%) especially with the use of additional criteria besides lymph node size, such as central necrosis and irregularity of the lymph node border, but their sensitivity is low (20- 40%), resembling performances in other malignancies.[9-11]

[18]F-fluorodeoxyglucose (FDG) positron emission tomography (PET) with non-contrast-enhanced low dose CT (further mentioned as FDG-PET/CT) has the ability to detect suspicious normal-sized lymph nodes and could therefore be more sensitive in detecting lymph node metastases than MRI or contrast enhanced CT alone.[12] It also provides whole body staging, which could be helpful for detection of distant metastases. A previous small series from our group showed its benefits for pelvic staging.[13-15] However, that small series of 18 patients excluded a substantial part of contralateral pelvic sides from the analysis and did not provide a per patient analysis.[15]

To ultimately improve patient selection for multimodal treatment, the present study evaluates the accuracy of FDG-PET/CT for pelvic and distant staging in a larger cohort of penile cancer patients at high risk of pelvic lymph node metastases.

PATIENTS & METHODS

Patients

This study was approved by the institutional medical ethical committee and the need for written informed consent was waived. We retrospectively analyzed all staging FDG-PET/CT scans performed in patients with advanced local or inguinal disease. Patients at the Netherlands Cancer Institute who underwent a staging FDG-PET/CT for penile cancer were included if they had:

1. Clinically high suspicion of inguinal lymph node metastases (N2-N3: palpable multiple unilateral, bilateral or immobile lymph nodes), and/or

2. Bulky primary tumor (bulky T3) or clinically T4 disease.

Patients that had underwent invasive staging of the groins (sentinel node procedure, excision biopsy or lymph node dissection) at the time of scanning, were excluded. PET/CT scans for staging disease recurrences were not excluded. All included patients had no previous treatment of the lymph nodes (i.e. no lymph node dissections, chemotherapy or radiotherapy before PET/CT).

FDG-PET/CT preparation and acquisition

Patients fasted for ≥6 hours and received oral prehydration before intravenous injection of 190-240 MBq FDG. One hour after injection, images were acquired with the patient in supine position. FDG-PET/CT was acquired on integrated PET/CT scanners (Gemini TF or Gemini TF Big Bore, Philips, Amsterdam, the Netherlands). No contrast agents were used. First, a low-dose CT scan (dose modulated, 40mAs, 2mm slice thickness) from the groins to the skull base was performed. Afterwards, a PET scan was made (2 minutes per bed position). Images were corrected for attenuation using the CT images and reconstructed with 4 mm isotropic voxels.

FDG-PET/CT interpretation

Each FDG-PET/CT scan was separately revised by two experienced nuclear medicine physicians (authors MV, MD and/or EV) blinded for patient data. It is important to note that only pelvic areas were studied, not the inguinal areas. Right and left sided pelvic lymph nodes and distant lesions were evaluated. An example of a PET-positive iliac node is provided in **Figure 1**. Pelvic sides and distant lesions were scored for clinical suspicion using a 4-point scale (**Table 1**). Scores 0 and 1 were regarded not suspicious, scores 2 and 3 were regarded suspicious

for metastasis. If discrepancy in scoring occurred between the two readers, a consensus reading was done. All scores (0-3) were included in the analyses.

Secondly, FDG-uptake of regions was scored using adjusted Deauville criteria (Supplementary **Table S1**).

For the interpretation of distant lesions, a distinction was made between distant metastases and additional findings. Additional findings were spots with increased uptake in locations unusual for penile cancer metastases and likely to be of other aetiology (or noise).
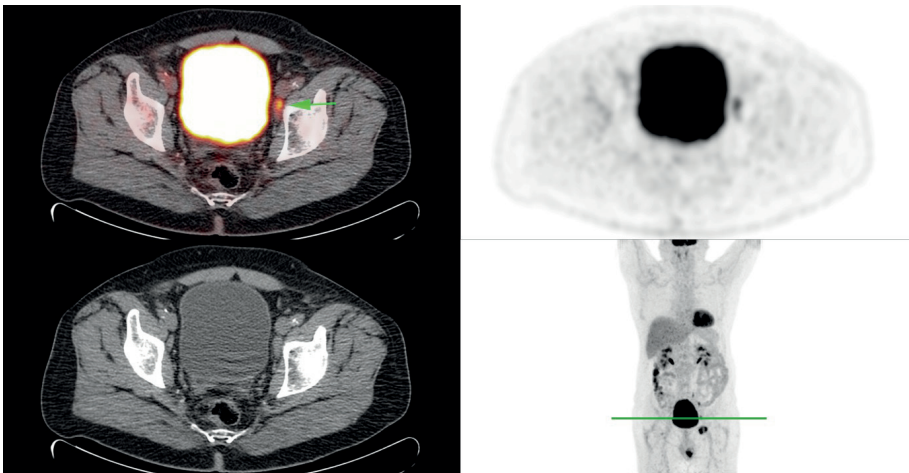

Figure 1. FDG-PET/CT images. Images of a patient with left iliac lymph node metastasis detected with FDG-PET/CT. Transaxial low-dose CT (bottom left), PET (upper right) and PET/CT fused (upper left) images, and coronal maximum intensity projection of PET (bottom right).

Table 1: Probability score for pelvic sides and distant lesions based on clinical suspicion

Score	Clinical suspicion (estimated risk of malignancy)
0	not at all suspicious (~10%)
1	probably benign (~30%)
2	probably malignant (~70%)
3	highly suspicious for malignancy (~90%)

Reference standard

PET findings were compared to histopathology after dissection, other imaging modalities, or to clinical follow-up of at least 1 year (with confirmed metastases or death due to disease within one year, or no evidence of disease for at least one year). Additional imaging (such as simultaneous contrast enhanced CT or follow-up imaging) was used as a reference for positive findings only. With clinical follow-up, metastases were considered to have been present at the time of PET if they became apparent within one year after PET. Absence of signs of disease for at least one year after PET was considered indicative for absence of disease at the time of PET.

For distant metastases observed on FDG-PET/CT, reference was cytological or histological proof, follow-up imaging, death of disease within a year (true positive detection), or clinical follow-up of at least one year without signs of disease (false positive detection).

Per patient scoring

Analyses were performed per pelvic side and per patient. A decision tree for per patient scoring is provided in **Figure 2**. In short, metastasis outcome was the dominant finding if present. Any false negative pelvic detection was dominant to other findings. Only patients with bilateral true negative findings were scored true negative.
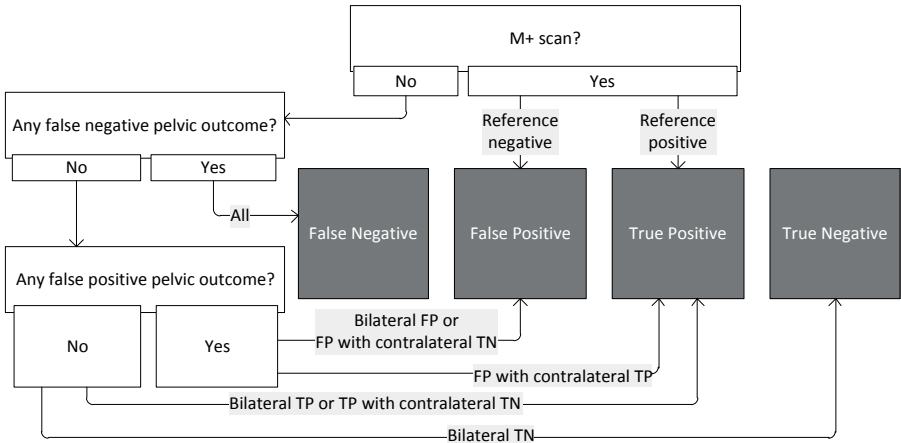

Figure 2. Decision tree for patient scoring. FP, false positive; FN, false negative; TP, true positive; TN, true negative.

Statistical analysis

Median follow-up was estimated with inversed Kaplan-Meier method. Interobserver agreement in score 0-3 between the nuclear medicine physicians before consensus was measured with quadratic-weighted kappa analysis in Stata Statistical Software (release 11, StataCorp. College station, Texas, USA). After consensus, scores 0-1 were regarded as negative, scores 2-3 as positive for metastases. A 2x2 table was constructed for pelvic and patient analysis. Test performance (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy) were calculated with 95% confidence intervals (CI) using R software (version 4.4.3, The R Project for Statistical Computing, R Core Team. Vienna, Austria).

For distant metastases, only positive findings were compared to the reference standard and the PPV was calculated.

Survival was estimated with the Kaplan-Meier method and the LogRank test using SPSS software (version 22, IBM, Armonk, USA).

RESULTS

Inclusion of patients

Between March 2006 and August 2016, a total of 177 potentially eligible penile cancer patients underwent FDG-PET/CT. Based on the inclusion criteria 80 patients were eligible. Six patients' scans were unavailable for revision (no images available, no CT images available or poor-quality images) and 13 patients were not included because no good reference was available at all. The remaining 61 patients were analyzed, their characteristics are summarized in **Table 2**. Of these, nine had no available reference for bilateral pelvic lymph nodes (but did have reference for patient-based analysis), and ten had only reference for one pelvic side. This resulted in a per-pelvis analysis of 94 pelvic sides in 52 patients. Eight of 61 patients were included in a previous analysis by Graafland et al.[15]

The indication for FDG-PET/CT scanning was clinical stage N2-3 in 46 cases, large primary tumor in 12 cases, and both in 3 cases.

Interobserver agreement

The interobserver agreement was high. Pelvic scoring based on clinical suspicion resulted in quadratic-weighted Kappa of 81.8% respectively. For metastases this was lower: 73.4%.

Table 2: Patient characteristics

Variable	Category	Patients (%) N=61
Indication for FDG-PET/CT *n (%)*	cN2/3	46 (75)
	Bulky T3/T4	12 (20)
	Both	3 (5)
Follow-up *Median (IQR)*	Months	52 (21 – 60)
Died within one year *n (%)*	Curative treatment	16 (26)
	Palliative treatment	6 (10)
	No treatment	4 (7)
Multimodal therapy *n (%)*	Neoadjuvant chemotherapy	12 (20)
	Palliative chemotherapy	3 (5)
	Chemoradiation	4 (7)
	Palliative chemoradiation	1 (2)
	Adjuvant radiation	3 (5)
	Palliative radiation	1 (2)
	Palliative surgery	1 (2)
Reference standard *n (%), total of 122*	PLND	44/122 (36)
	Positive imaging*	17/122 (14)
	Negative follow-up	33/122 (27)
	No reference	28/122 (23)

IQR, interquartile range; PLND, pelvic lymph node dissection.

* Imaging of 17 pelvic sides (in 11 patients) consisted of follow-up FDG-PET/CT in seven patients, concurrent or follow-up CT in one patient, a sequence of both in two patients, and MRI in one patient.

Staging of pelvic lymph nodes

The results of pelvic staging with FDG-PET/CT are depicted in **Table 3**. Of 94 analyzed pelvic sides, 33 showed pelvic nodes on FDG-PET/CT of which 28 were confirmed (14 with PLND, 14 with additional imaging). Fifty-one pelvic sides did not show positive nodes on FDG-PET/CT. Five of which later proved false negative (all unilaterally, further described in Supplementary **Table S3**), and twenty of which proved true-negative on prophylactic PLND. Thirteen cases had a false-positive result on one or both sides. Overall, accuracy of FDG-PET/CT per pelvis was 79% (sensitivity 85%, specificity 75%, PPV 65%, NPV 90%).

These analyses include fourteen patients that had neoadjuvant chemotherapy or chemoradiation between FDG-PET/CT scanning and reference. Their

results included seven false-positive findings and three true negative findings that are possibly skewed by the therapy. Without these fourteen patients, test performance is slightly better (N=38, sensitivity 86%, specificity 84%, PPV 69%, NPV 93%).

Table 3: Results and calculated test performances of FDG-PET/CT according to the clinical suspicion score

A. Cross table FDG-PET/CT per pelvis

	Reference+	Reference-	total
PET+	28	15	43
PET-	5	46	51
total	33	61	94

B. Per patient

	Reference+	Reference-	total
PET+	30	10	40
PET-	6	15	21
total	36	25	61

C. Calculated test performances of FDG-PET/CT for pelvic lymph node staging

	Per Pelvis (%)	Per patient (%)
Sensitivity	85	83
Specificity	75	60
NPV	90	75
PPV	65	73
Accuracy	79	74

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

Detection of distant metastases

Fourteen patients had lesions suspicious for distant metastases, of whom 13 were confirmed (PPV 93%). Lesions described as metastases were mostly abdominal/thoracic lymph nodes or lung lesions (both n=7).

Additional findings occurred in 17 patients. These findings mostly concerned foci in the gastrointestinal tract (n=5, considered to be most likely due to imaging noise) or liver (n=5), and lymph nodes in patterns atypical for metastases (e.g. sarcoid-like mediastinal nodes, n=3).

Considering the whole patient

Based on patient's bilateral pelvic and distant outcomes, a per-patient result was scored (**Figure 2**). False positive outcomes were found in 6 patients, true negative outcomes in 15 patients. Sensitivity was 83%, specificity 60%. PPV was 73%, NPV 75%, and overall accuracy was 74% (**Table 3**). Leaving out the patients with neoadjuvant chemotherapy and curative CRT, specificity improved to 71% (NPV to 79%, PPV and accuracy to 78%).

Overall survival

Median follow-up was 52 months. Evaluation of overall survival was done for three groups of patients: 1) those without suspicion of pelvic or distant metastases, 2) those with suspicion of pelvic but not distant metastases, and 3) those with suspicion of distant metastases. The estimated median survival was 45, 34 and 8 months respectively, Log Rank p<0.01 (**Figure 3**).

FDG uptake (adjusted Deauville criteria)

The FDG uptake score of lesions using adjusted Deauville criteria (Supplementary **Table S1**), gave similar sensitivity and NPV as the visual analysis described above (clinical suspicion score, **Table 3C**; Supplementary **Table S2**). The FDG uptake score led to more false-positive results (20 vs. 15) and one less false-negative result (**Table 3,** Supplementary **Table S2**. For pelvic staging based on FDG uptake score, accuracy was 74%, sensitivity 89%, and specificity 67% (Supplementary **Table S2**).

DISCUSSION

We studied FDG-PET/CT performance for staging pelvic lymph nodes in penile cancer patients at significant risk for pelvic metastases. The rationale being able to start multimodality treatment as early as possible and avoiding unnecessary surgery. It is important to note that FDG-PET/CT cannot replace invasive staging of the inguinal lymph nodes, and therefor, only pelvic areas were studied.

We found good test performance, especially regarding sensitivity and negative predictive value (83-85% and 75-90% respectively). Also, FDG-PET/CT accurately identified 10 cases (16%) with distant metastases, and FDG-PET/CT positivity was associated with decreased survival. The adjusted Deauville criteria performed similarly to the clinical suspicion score and may serve as a reference for PET imaging analysis; nodes with higher FDG uptake than the blood pool can be regarded malignant, albeit with more false-positive detections.

Our study shows that FDG-PET/CT is an accurate non-invasive staging modality for pelvic lymph nodes for patients at high risk of pelvic metastases. Its sensitivity is much higher than sensitivities published for contrast enhanced CT and MRI.[16,17] In addition, distant metastases are visualized on FDG-PET/CT in a significant percentage of patients, with a high PPV.
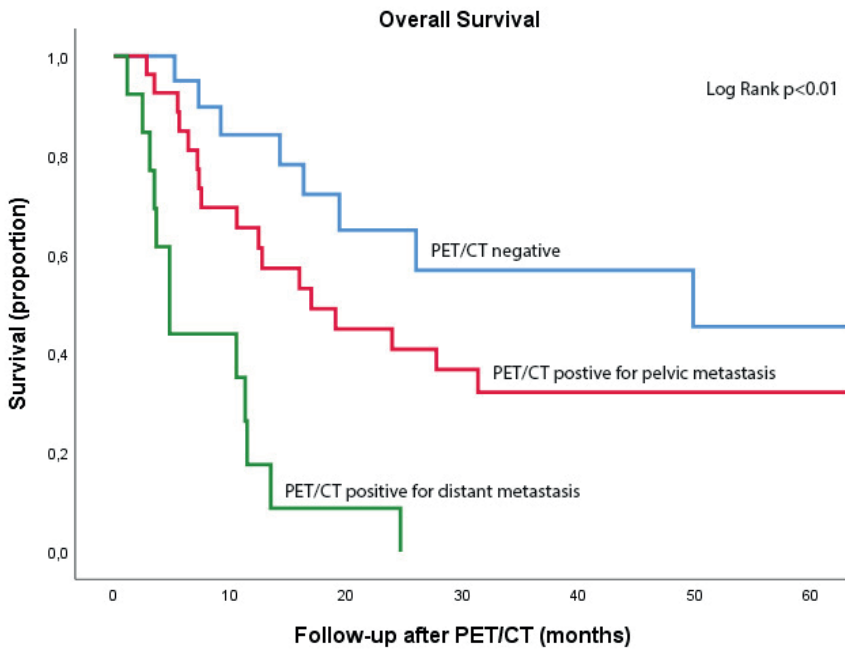

Figure 3. Survival after FDG-PET/CT

Nº at risk	0 months	20 months	40 months	60 months
Negative	20	9	6	2
Pelvic+	27	11	7	3
M+	14	1	0	0

Overall survival of patients without suspicion of pelvic or distant metastases (blue line), patients with suspicion of unilateral or bilateral pelvic metastases (red line) and patients with suspicion of distant metastases (green line). Log Rank test p <0.01.

Given the suboptimal specificity, clinical implementation of FDG-PET/CT will expectedly result in a number of false-positive detections with subsequent overtreatment. Adverse consequences of false negative FDG-PET/CT will be infrequent, given the high sensitivity and NPV.

The suboptimal specificity (60-75%) in this study contrasts to a previous series of our institute by Graafland et al. that found a specificity of 100%, possibly explained by our larger sample size. Zhang et al. also studied FDG-PET/CT for detection of metastases[21]. In a per-lymph node analysis irrespective of location, they found a higher sensitivity and specificity than we did. This may be due to their inclusion of inguinal node staging in patients already suspected of inguinal metastases. In a per patient analysis, they reported a similar sensitivity (81%), but a higher specificity (93% vs. 60% in the present series). This can be explained by their scoring system; Zhang et al. classified all patients as true positive with one true positive lesion, even if another lesion was false positive or false negative. In our analysis, false negative findings on one side were dominant, except for positive distant metastatic findings.

In other malignancies, several studies investigating FDG-PET/CT for pelvic lymph node staging reported a higher accuracy than contrast enhanced CT alone.[16,17,22,23] In penile cancer, this has not been shown before.

A meta-analysis studied FDG-PET/CT for groin assessment in penile cancer.[24] In clinically node-negative groins, the pooled sensitivity of FDG-PET/CT was only 57%, but in patients with palpably enlarged nodes this increased to a pooled sensitivity and specificity of 77% and 81% respectively after Egger's correction for publication bias.[24]

This study is not devoid of limitations. Its retrospective nature has inherent biases that cannot be accounted for. During the years, not all patients with clinical stage N2-3 or large primary tumors underwent FDG-PET/CT before invasive staging. Our study possibly includes the most suspicious cases, that possibly led to higher positive predictive values. Also, we did not exclude patients that underwent either curative or palliative chemotherapy or chemoradiation. Some of the results in these patients (false-positive and true-negative) may be skewed by therapy effect. Thirdly, patients with residual or intercurrent local or inguinal disease were not excluded; in very few patients, new metastases that developed in the year following FDG-PET/CT may have caused false-negative findings. Lastly, the gold standard (pathology) was not performed in all patients. Hence, the reference standard is based on a combination of tests (pathology, imaging and

follow-up) with possible influences on the outcomes. Also, no direct comparison could be made with contrast enhanced CT or MRI since only a low dose CT for attenuation correction was available. These results should be prospectively validated including a comparison to contrast-enhanced CT.

Nonetheless we advocate the use of FDG-PET/CT for staging patients with clinical stage N2-N3 and/or bulky stage T3 or T4 primary tumors. In these high-risk patients, FDG-PET/CT provides adequate non-invasive staging of pelvic lymph nodes, guiding treatment choices for multimodal therapy. In other words, FDG-PET/CT can select patients with pelvic metastasis for e.g. neoadjuvant chemotherapy or chemoradiation. It also provides timely detection of distant metastases with consequent omission of futile lymphadenectomies.

CONCLUSIONS

FDG-PET/CT has a high sensitivity and moderate specificity for pelvic staging, and high PPV for distant staging in penile cancer patients at risk of pelvic metastases. We advocate implementation of FDG-PET/CT in clinical practice for staging these patients and improvement of patient selection for multimodal therapy.

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Acknowledgements
We would like to thank our colleagues from the radiology archive of our institute for retrieving scans from the archive that were primarily inaccessible.

Compliance with ethical standards
This study was conducted in accordance with institutional ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the medical ethical committee of our institute.

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Supplementary Table S1: Lesion score systems based on intensity of FDG uptake (adjusted Deauville criteria).

Score	FDG uptake
0	none
1	≤ blood pool
2	> blood pool ≤ liver
3	> liver

FDG, ^{18}F-fluorodeoxyglucose.

Supplementary Table S2: FDG uptake score: results & calculated test performances of FDG-PET/CT

A. Cross tables of PET-results and reference outcome (per pelvis)

Per pelvis	Reference+	Reference-	total
PET+	29	20	49
PET-	4	41	45
total	33	61	94

B. Cross tables of PET-results and reference outcome (per patient)

Per patient	Reference+	Reference-	total
PET+	30	13	43
PET-	5	13	18
total	35	26	61

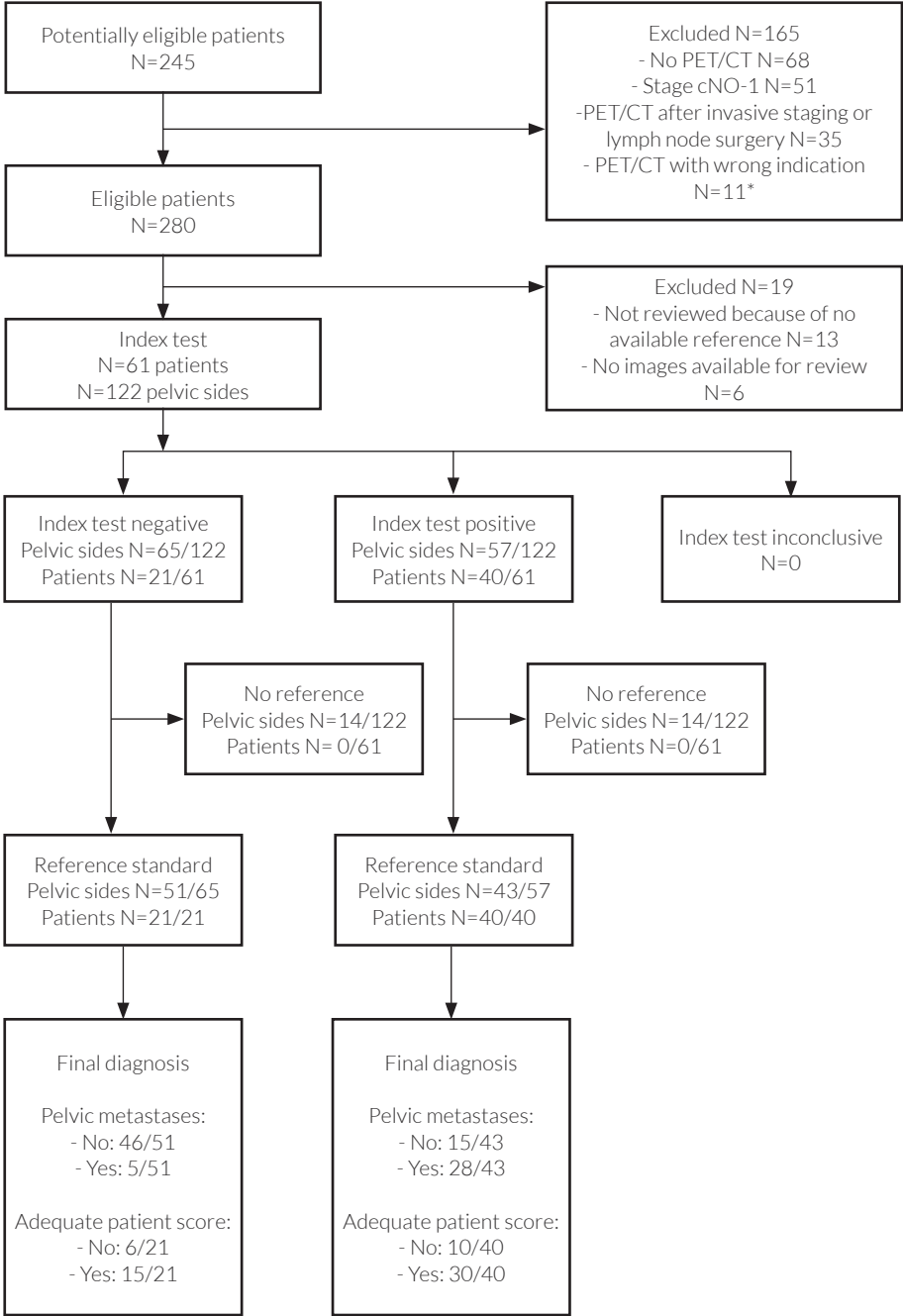**C. Calculated test performances of FDG-PET/CT for pelvic lymph node staging using the FDG uptake score**

	Per Pelvis (%)	Per patient (%)
Sensitivity	89	86
Specificity	67	50
NPV	91	72
PPV	59	70
Accuracy	74	70

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

Supplementary Table S3: Characteristics of patients with false negative results

Case no	FN in pelvic / patient analysis	Left / Right	Lymph node ratio (positive/ total) at pathology	Largest size of metastasis	Time from FDG-PET/CT to reference	Remarks
8	Pelvic	Right	NA	Not reported	Died within a year	Surgically treated. Extensive metastasis within six months of follow up.
74	Pelvic	Right	NA	4.3cm on MRI	9.6 months	Irradical resection of basaloid primary T4 tumour
81	Pelvic	Left	2/5	Not reported	0.9 months	Treated with chemoradiation.
84	Pelvic	Right	NA	Not reported	5.1 months	Residual or recurrent tumour of the penis was seen on follow-up PET/CT after surgical treatment.
91	Pelvic	Right	1/10	4mm at pathology	1.1 months	Node was visible on PET/CT, but regarded as not suspicious.
82	Patient only	NA	NA	Lymphangitis carcinomatosa subcutaneous in hip area	Died within a year	Surgically treated.

FN, false negative; NA, not applicable.

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┌─────────────────────────────┐              ┌─────────────────────────────┐
│  Potentially eligible patients │             │  Excluded N=165             │
│         N=245                │              │  - No PET/CT N=68           │
└─────────────────────────────┘              │  - Stage cNO-1 N=51         │
              │                              │  -PET/CT after invasive staging or │
              │──────────────────────────▶  │  lymph node surgery N=35    │
              │                              │  - PET/CT with wrong indication │
              ▼                              │         N=11*               │
┌─────────────────────────────┐              └─────────────────────────────┘
│  Eligible patients          │
│         N=280               │
└─────────────────────────────┘              ┌─────────────────────────────┐
              │                              │  Excluded N=19              │
              │──────────────────────────▶  │  - Not reviewed because of no │
              │                              │  available reference N=13   │
              ▼                              │  - No images available for review │
┌─────────────────────────────┐              │         N=6                 │
│  Index test                 │              └─────────────────────────────┘
│  N=61 patients              │
│  N=122 pelvic sides         │
└─────────────────────────────┘
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Index test negative	Index test positive	Index test inconclusive
Pelvic sides N=65/122	Pelvic sides N=57/122	N=0
Patients N=21/61	Patients N=40/61	

No reference	No reference
Pelvic sides N=14/122	Pelvic sides N=14/122
Patients N= 0/61	Patients N=0/61

Reference standard	Reference standard
Pelvic sides N=51/65	Pelvic sides N=43/57
Patients N=21/21	Patients N=40/40

Final diagnosis	Final diagnosis
Pelvic metastases:	Pelvic metastases:
- No: 46/51	- No: 15/43
- Yes: 5/51	- Yes: 28/43
Adequate patient score:	Adequate patient score:
- No: 6/21	- No: 10/40
- Yes: 15/21	- Yes: 30/40

Supplementary Figure 1.
* examples are: response evaluation PET/CTs, PET/CT because of other malignancies, or because of a suspicion of M+

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Chapter 7

Surgical and oncological outcomes in patients after vascularized flap reconstruction for loco-regionally advanced penile cancer

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ABSTRACT

Background: Treatment of locoregionally advanced penile cancer (LAPSCC) is challenging. The exact role (in terms of oncological benefit) of extensive surgery is not well established. Moreover, surgery invariably leads to large defects requiring reconstructive surgery. Rectus abdominis myocutaneous (RAM) and abdominal advancements flaps have independent and constant blood supply, are easily harvested, and provide substantial skin coverage and soft tissue.

Objective: To determine the surgical and oncological outcome in patients with LAPSCC undergoing surgical resection with RAM flaps.

Design, Setting and Participants: From 2002-2016, a multi-institutional database identified fifteen LAPSCC patients undergoing flap reconstructions.

Intervention: Local surgical resection with RAM or abdominal advancement flap reconstruction.

Outcome measurements and Statistical analysis: Perioperative and pathologic data were collected. Postoperative complications were identified using the Clavien-Dindo classification of surgical complications.

Results and limitations: Fifteen patients (median age: 61) were treated; ten with curative intent. Thirteen patients received induction chemotherapy. Thirteen of 15 patients (87%) experienced wound complications including five Clavien-Dindo grade III complications. In 11/15 patients (73%) disease recurred (median recurrence-free interval: 106 days). The majority of recurrences (91%) were locoregional, four of which also had lesions in distant organs. Ten of 15 (67%) died of disease. Overall median follow-up interval was 10.5 months. This study was limited by its retrospective design, absence of quality-of-life measurements, and cohort size.

Conclusions: The results of this study show that surgical resections with reconstruction is associated with a risk of perioperative complications, including high grade according to Clavien-Dindo classification. With a cure-rate of 27%, surgery needs careful consideration, with a need for alternative treatments. Also lack of robust quality-of-life-data is a serious shortcoming in the decision process for this patient category.

Patient summary: Surgery in locoregionally advanced penile cancer has a low cure rate. Reconstruction of defects is surgically feasible, albeit with a high risk of complications. Further, decision making lacks robust data on quality-of-life after surgery.

INTRODUCTION

Treatment of extensive locoregional penile cancer is challenging. Some patients delay treatment and seek care only when disease is far advanced with extensive local and/or regional involvement. These lesions may lead to large fungating masses with destruction of skin and soft tissue. These patients have poor quality-of-life because of pain, fetor, lymphedema, lymphorrhea and infection, that may leave the patient bedridden, debilitated and difficult to manage by family and/or health workers. Surgery has been used in the past to alleviate complaints of large tumours deposits, and in some cases, to attain cure. However, the exact role of surgery in locally advanced disease, either palliative or curative, is not well known. Moreover, surgical removal of all tumour deposits leads invariably to large wound defects **(Figures 1 and S1)** necessitating reconstructive surgery in collaboration with plastic and reconstructive surgeons.

Even if cure turns out to be an unattainable goal, surgery for palliative motives can be considered.[1] Primary coverage with a skin graft is usually impossible with exposed bones, nerves, fascia and vessels as it lacks vessels for skin graft ingrowth. Therefore, vascularized reconstructive techniques like myocutaneous and advancement flaps are first choice. Advantages include independent and constant blood supply, ability to harvest in supine position, adequate bulk to obliterate dead space and provide protection to femoral vessels, ease of closure of the donor site and additional skin to allow tension free closure of the defect.[2,3] Additionally, this technique is of value in salvage procedures following previous surgery or radiation therapy.

The surgical management of advanced penile carcinoma has only been reported in small series with a maximum of five patients, summarized in **Table 3**.[3-13] In this study, we aim to evaluate surgical and oncological results in a two-institutional experience, including fourteen patients who underwent flap reconstruction after surgery for advanced locoregional penile carcinoma in both palliative and curative settings.
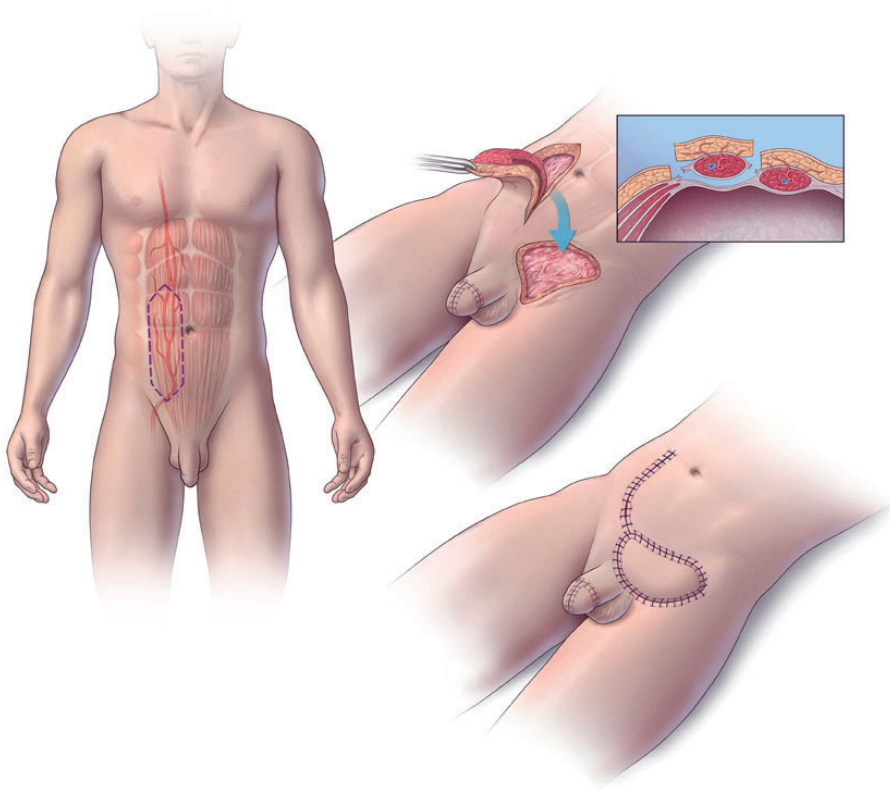
Figure 1: Anatomy of Rectus Muscle and Illustrating Flap Anatomy (made with Adobe PhotoShop)

PATIENTS AND METHODS

Patients

Between September 2002 and December 2016, 15 patients were seen at two referral centres with extensive locoregional disease, in whom resection would leave a defect that required reconstructive surgery. All patients were identified from a prospectively kept database, staged according to the 2009 TNM classification of penile cancer and retrospectively analysed.[7]

Surgical treatment

All patients had extensive surgery with removal of inguinal and/or penile skin, inguinal and sometimes pelvic lymphatic tissue, further specified in **Table 1**. In

patients treated with palliative intent, the main goal was to obtain local control, loss of pain, fetor and easy management of the local situation.

The operative technique of VRAM, ORAM (vertical or oblique rectus abdominis myocutaneous flap) or abdominal advancement was performed in collaboration with a plastic surgeon. He/she decided which reconstructive technique was used and performed the reconstructive procedure. The patients were able to remain in the supine position throughout the procedure. After preparation of the recipient site the defect was measured (**Figure S1**) and an appropriately sized flap was marked on the skin of the ipsilateral or contralateral rectus abdominis, depending on location and size of the defect. Flaps were incised through skin, subcutaneous tissue, anterior rectus sheet and muscle. After identification and preparation of the inferior epigastric artery (for an increased range of motion; **Figure S2**) the flaps were mobilized (**Figure 2A**) and transferred to the recipient area (**Figure 2B**). VRAM flaps use the rectus abdominis muscle with the skin island directly on the muscle. ORAM flaps leave more of the muscle in situ. Their skin paddle is taken in oblique direction, based on the umbilical perforating vessels. VRAM flaps have excellent blood supply and are easily adjusted to the requested size. ORAM flaps have lower donor site morbidity, but the skin island partially depends on communicating perforating vessels. **Figure 3** shows VRAM donor site re-approximation with a biological mesh (**Figure 3A**) and with a knitted monofilament polypropylene mesh (**Figure 3B**). In all patients undergoing inguinal surgery, low suction drainage was placed in the inguinal defect.

Post-operative care

Subcutaneous prophylaxis for deep vein thrombosis was started post operatively. Patients were kept in bed for at least 24 hours. Drains were removed when output decreased to ±50 mL per day.

Outcome Measurements and Statistical Analysis

Surgical results were measured in terms of perioperative and postoperative complications, classified according to the Clavien-Dindo classification of surgical complications.[14] As oncological outcomes, we reported resection margin status, disease recurrence, whether it recurred within the resected site, recurrence-free survival (RFS) and disease-specific mortality (DSM). Pain was measured on a visual analogous scale (VAS). This study was done in accordance with institutional ethical guidelines, based on good clinical practice.
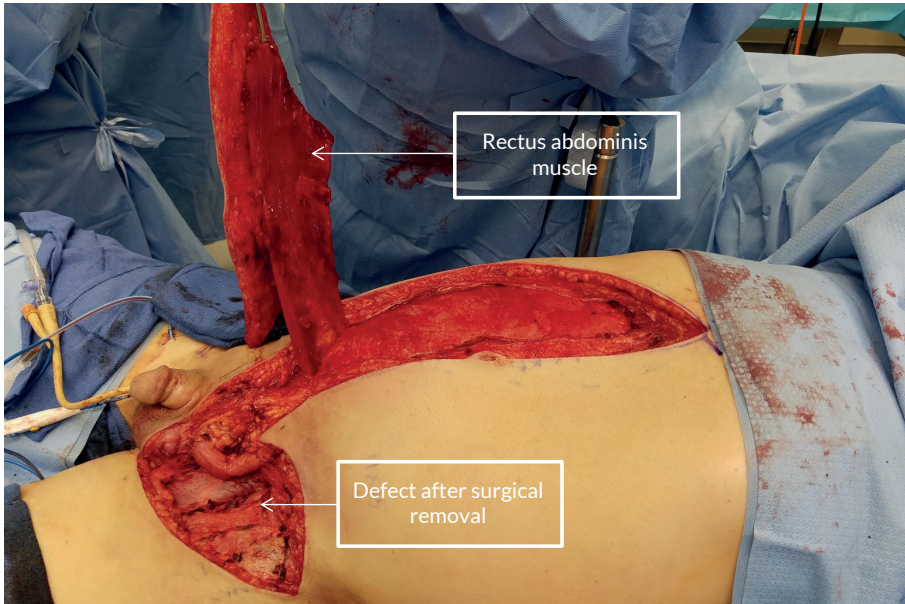
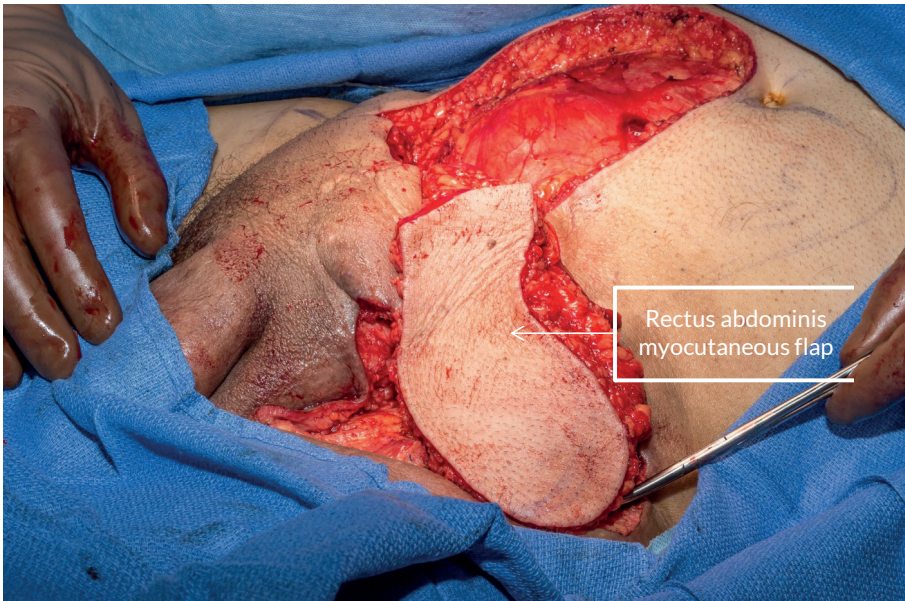**Figure 2A.** Free flap prior to tunneling to defect.

Figure 2B. Rectus Flap is rotated with inferior pubic attachments and skin attachments released.
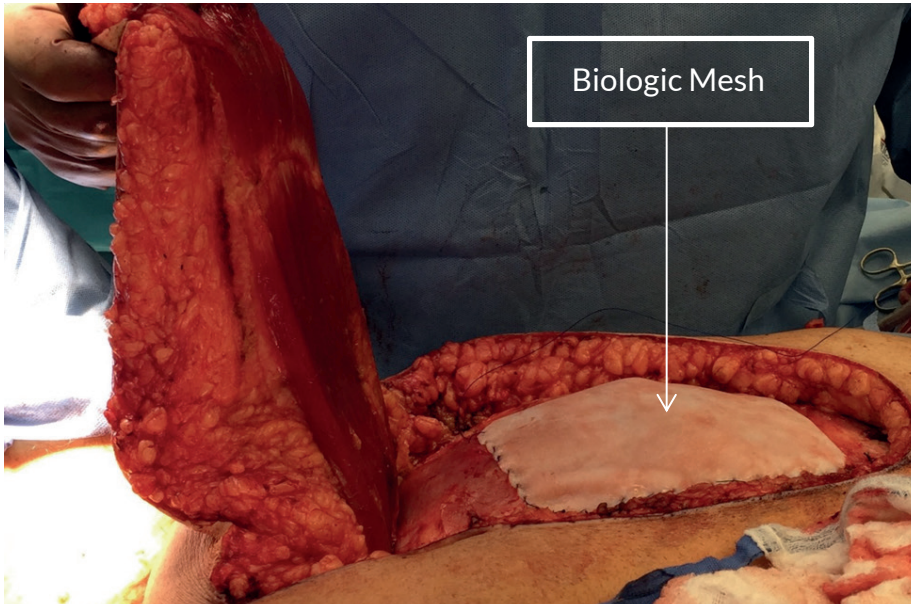
Biologic Mesh

Figure 3A. Demonstration of Biologic Mesh placement in Rectus Defect.

Figure 3B. Demonstration of Knitted Monofilament Polypropylene Mesh placement in Rectus Defect.

RESULTS

Patients

Fifteen patients (median age 61 years (range: 39-85) are described in **Table 1**. All had squamous cell carcinoma (SCC) of the penis. Eight of these patients (53%) presented with locoregionally advanced primary disease and seven (47%) had locoregional recurrences. Thirteen patients received induction chemotherapy prior to surgery. Five patients (33%) were operated with palliative intent only, as they progressed through chemotherapy. The remaining ten men (67%) underwent operation with curative intent. Postoperative radiation was administered to eight patients (53%), three of which were palliative cases, in order to improve local control. Wounds had to be healed before radiation was started.

Ten patients (67%) underwent VRAM-flap reconstruction, in four cases combined with other reconstructive techniques such as abdominal advancement (**Table 1**). Four patients (27%) underwent ORAM, and one patient (7%) abdominal advancement only.

Surgical results

All but one flaps remained vital. One flap slowly became ischemic and necrotic despite no signs of entrapment per-operatively. Initially, this flap was managed conservatively, but needed necrotectomy on day 11 after surgery. Five of 8 patients that underwent adjuvant radiotherapy, received it elsewhere. Reports from those centers, and clinical follow-up indicated that all flaps remained vital during radiation.

Twelve of 15 patients (80%) experienced wound complications, including five grade III complications (**Table 1)**. One patient experienced a postoperative ileus and two patients had a delirium as general complications additionally to their wound complications. All complications could be managed conservitavely unless stated otherwise in **Table 1**.The median hospital stay was 13 days (range: 4 – 57 days). All but one patients were discharged to home, patient 7 was discharged to a nursing home.

Of the palliative surgery group, pain was evaluated by Visual Analogue Scale (VAS) in four patients. In two patients, the VAS was reduced by 5 points between presentation and day of discharge. The pain of the third patient was reduced from unknown VAS to VAS 1 during reduced analgesics use. The fourth patient reported no pain at presentation, but was operated to relief managerial problems related

to his large inguinal fungating mass. The fifth patient was operated because bulky nodes were encasing the femoral vessels.

Oncological results

Median follow-up was 10.5 months (range: 1.7 to 74.2 months). During that time, disease recurred in eleven of fifteen patients (73%; **Table 2**). The median RFS was 106 days after resection (3.5 months, range: 9 – 556 days). Three patients (20%), two of which were palliative cases, experienced cutaneous metastatic disease in or around the reconstructed skin before postoperative radiotherapy could be given, one additional patient after adjuvant radiation was given. Another patient had preoperative clinical evidence of cutaneous metastasis, that recurred clinically only 9 days postoperatively. The other recurrences included locoregional recurrences, as well as a groin metastases in non-dissected areas. Five cases had distant metastases.

Ten patients, all with disease recurrences, died of disease after a median time of 4.7 months (range: 1.7-23.0 months) from resection. This resulted in a DSM of 67%. Four men had no evidence of disease at follow-up, with median follow-up in these men of 54.6 months, ranging from 8 to 74 months. All four underwent an excision with tumour negative margins. On the other hand, four of eight patients with tumour-free margins had recurrent disease, and three died of their disease. One had histopathologic evidence of lymphangitis carcinomatosis, two died of a recurrence with distant spread, one is still alive after having a regional recurrence and an perirectal mass.

DISCUSSION

The management of extensive locoregional disease of penile cancer is challenging. Three aspects have to be considered: technical aspects of resection and reconstruction, oncological aspects (recurrence-free survival and disease-specific survival), and effect on palliation.

Technically, complete resection of extensive disease was not always feasible, despite wide local excision. Pre-operative determination of surgical feasibility by physical examination in combination with imaging is not always easy. Our results emphasize the difficulty in judging oncologically sound resection pre- and per-operatively. MRI could aid planning of resection and prediction of resectability. Reconstruction could always be performed, but not without complications.

Table 1: Patient characteristics and wound complications of curative and palliative cases

Center	Pt	Age	BMI	ASA	cTNM	iChT	Surgical Treatment	Reconstructive Surgery	Postoperative Complication	Description
Curative intent										
NCI	1	58	27	2	rT4N0M0	Yes	Resection local recurrence, including prostate / urethra / part of symphysis	VRAM-v for central defect + Mitrofanoff procedure	Grade I	Ileus
NCI	2+	61	29	2	T3N2M0	Yes	Total penectomy and bilateral ILND + bilateral orchiectomy + subtotal scrotectomy	Abdominal advancement flap for central defect	Grade I	Wound dehiscence
NCI	3	69	19	2	T4N0M0	Yes	Total penectomy + bilateral ILND	VRAM-v for central defect + abdominal advancement flaps for lateral defects	Grade IIIb	Necrosis and loss of flap, requiring re-operation 3x, incl TFL flap
NCI	4	76	21	3	T4N3M0	No	Total penectomy + bilateral ILND + PLND	VRAM-v for central defect	Grade II	Wound infection requiring oral antibiotics
NCI	5	63	24	2	T4N3M1	Yes	Total penectomy + bilateral ILND	ORAM-v for central defect	No	N/A
NCI	6	68	30	1	rT4N0M0	Yes	Corpus cavernosus + pubic bone resection	ORAM-v for central defect	Grade IIIa	Abscess requiring drainage and antibiotics
MCC	11	59	-	-	T3N2M0	Yes	Total penectomy + bilateral ILND	VRAM-f for right defect + Sartorius muscle transposition for left defect	Grade I	Right foot drop secondary to peroneal nerve injury
MCC	13	57	32	2	rT2N2M0	Yes	Previous bilateral surgery with Sartorius muscle transposition; Salvage left ILND	VRAM-v for left defect	No	N/A

MCC	14	46	25	2	T3N2M0	No	Previous locoregional surgery; Total penectomy + simple cystectomy + bilateral cutaneous ureterostomies	VRAM-v for central defect	IIIb	Wound dehiscence requiring flap revision
MCC	15	52	29	2	T4N0M0	Yes	Previous locoregional surgery; Radical perineal resection + bilateral partial ischial bone resection	VRAM-v for central defect	II	DVT
Palliative intent										
NCI	7	78	21	2	rTxN3M0	Yes	Bilateral ILND + PLND right	VRAM-v for central/right defect + Sartorius muscle transposition for defect right	Grade IIIb	Necrosis, requiring re-operation, delirium
NCI	8	39	32	3	rT1N3M0	Yes	Bilateral ILND + right PLND + right orchiectomy	VRAM-f for left + TFL-v for right defect	Grade I	Wound dehiscence with edge necrosis
NCI	9	67	27	2	T2N3M0	Yes	Partial penectomy and bilateral ILND + PLND	ORAM-v for left defect	Grade I	Wound dehiscence
NCI	10	85	27	2	rTxN3M0	Yes	Total penectomy + left ILND + left orchiectomy	ORAM-v for left defect	Grade I	Wound dehiscence, delirium
MCC	12	47	29	2	rT3N2M1	Yes*	Partial penectomy + bilateral ILND + right PLND	VRAM-v for central defect	Grade IIIa	Abdominal wall abscess requiring drainage and antibiotics

CI, Netherlands Cancer Institute; MCC, Moffitt Cancer Center; Pt, patient; BMI, body mass index; ASA, ASA Physical Status Classification System; iChT, induction chemotherapy; ILND, inguinal lymph node dissection; VRAM, vertical rectus abdominis myocutaneous flap; ORAM, oblique rectus abdominis myocutaneous flap; TFL, tensor fasciae latae muscle flap; -v, vascularized; -f, free flap; DVT, deep venous thrombosis.

† this patient had reconstruction as a secondary procedure 50 days after primary surgery

* this patient underwent induction chemoradiation.

The VRAM flap, with its dual blood supply, has shown to represent an excellent means to achieve wound coverage with low morbidity following resection of advanced penile carcinoma in a small series of four patients.[9] In that study, postoperative morbidity was low and improvement of quality–of-life was reported. In a similar series of five patients with penile SCC undergoing VRAM flap, only one patient experienced donor site wound edge necrosis.[5] Likewise, Kuntscher et al. published a series of fifteen VRAM patients, one of whom had advanced penile SCC and suffered no major complications, only minor abdominal bulging (as mesh was not used at the donor site).[10] However, our findings do not mirror those above. Most patients experienced wound complications, including three who required additional surgery (debridement for wound necrosis, flap revision and flap loss). A plausible explanation is that the extent of disease and surgery are not comparable between both studies. **Table 3** illustrates a summary of the available literature on VRAM flaps in advanced penile cancer, including the findings of the present series.

In terms of oncological control, a minority of the group (four men) treated with curative intent had long lasting recurrence-free survival and disease-specific survival. In the patients undergoing palliative surgery, recurrence-free survival was disappointing (one developed a regional skin recurrence 9 days postoperatively).

Our series show predominantly local and regional recurrences after extensive surgery and induction chemotherapy. Five patients developed distant metastasis. This is somewhat in contrast to another study, which reported early death from distant metastasis (two out of four patients, with shorter follow-up).[9] In general, SCC has a strong tendency for locoregional growth. Penile cancer is no exception, and our patient series reflects this predilection.

Probability of local and regional recurrence is understandably higher when tumour-negative margins cannot be obtained. Unfortunately, as in the whole field of surgical oncology, negative surgical margins are no guarantee for cure, as half of the patients with tumour-free margins eventually had recurrent disease (albeit all after more than three months).

Additionally, after irradical resection, adjuvant radiotherapy, started after the surgical wounds have closed, can improve local control. Two patients had a relatively long recurrence free survival. Clinical signs of lymphangitis carcinomatosa or satellite skin lesions are ominous with a very high likelihood

Table 2: Disease course of all patients

Pt	pTNM	Histology	Tumor-free Margins	Postoperative RT	Hospital Stay (days)	Recurrence-free Survival° (days)	Type of Recurrence	Reconstruction Site	Follow-up (months)*	Status
Curative intent										
1‡	yrpT4NxMx	SCC grade 2	Yes	No	21	111	Inguinal + skin metastasis	No	12.1	DOD
2	ypT4N1Mx	SCC grade 3	No	No	4	68	Lung metastasis	No	3.5	DOD
3	ypT4N0Mx	SCC grade 3	No	Yes	57	104	Local recurrence scrotum, pubic region and skin	Yes	10.5	DOD
4	pT3N0Mx	SCC grade unknown	Yes	No	29	No recurrence	N/A	N/A	74.2	NED
5	ypT2N0Mx	SCC grade 2	Yes	No	11	No recurrence	N/A	N/A	65.3	NED
6	yrpT4N0Mx	SCC grade 2	No	Yes	6	556	Inguinal recurrence	No	22.6	DOD
11	ypT3N2Mx	SCC grade 2	Yes	Yes	8	140	Inguinal recurrence + liver metastasis	No	23	DOD
13	ypT2N3Mx	SCC grade 2	Yes	No	7	No recurrence	N/A	N/A	44	NED
14	pT3N2Mx	SCC grade 3	Yes	Yes	13	No recurrence	N/A	N/A	8	NED
15	ypT4N0Mx	SCC grade 3	Yes	Yes	6	226	Inguinal and perineal recurrence + perirectal mass	Yes	16	AWD
Palliative intent										
7##	yrpTxN3Mx	SCC grade 2	No	Yes	17	56	Local recurrence scrotum + skin	No	3	DOD
8##	yrpTxN3Mx	SCC grade 3	No	No	18	39	Local recurrence skin	Yes	1.9	DOD
9	ypT2N3Mx	SCC grade 2	Unlikely	Yes	14	137	Local / regional recurrence + lung metastasis	No	5.4	DOD
10±	yrpT2N3M1	SCC grade 3	No	Yes	14	9	Regional recurrence skin	Yes	1.7	DOD
12	ypT3N2Mx	SCC grade 2	Yes	No	6	106	Pelvic recurrence + bone and lung metastases	No	4	DOD

° Calculated from date of oncological resection until date of recurrence

* Calculated from date of oncological resection until last day of follow-up / death

‡ Patient had evidence of lymphangitis carcinomatosis at histopathologic examination

Both patients had clinical evidence of lymphangitis carcinomatosis at surgery

± Patient had clinical evidence of cutaneous metastases before surgery

DOD, death of disease; NED, no evidence of disease; AWD = alive with disease; N/A, not applicable

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of local recurrence, even in patients with response to chemotherapy or tumour-free margins at surgery. These patients are poor candidates for extensive surgery, as the meagre benefits, if any, do not outweigh such surgical procedure and it's complications.

Meaningful palliation was hard to determine in our series. However, absence of local recurrence can likely be considered a meaningful palliation, as suffering from local problems, such as pain, discharge and fetor, can be considerable and quite difficult to manage.[1] Hence, in terms of pain management, some palliation is achieved. However, considering the short life expectancy, this patient category likely warrants other than surgical treatment. Although pain was measured in the palliative cases, a robust quality-of-life analysis is lacking. Therefore it remains unknown how helpful these interventions truly were for these patients.

As oncological results are disappointing in our series, a re-evaluation of the role of surgery in locoregionally advanced penile tumours is suggested. Especially in patients where no tumour-free margins can be obtained based on presence of extensive nodal metastases, signs of lymphangitis carcinomatosa or satellite skin lesions. Based on our series, surgical resection and reconstruction can be attempted at least in patients in whom, by all available means, tumour free margins seem achievable. Otherwise enthusiasm for surgery needs to be mitigated. Our results underline the need of a realistic evaluation of survival odds with surgery, alternative treatments, and earlier involvement of palliative care.

Other treatment modalities are currently available. Chemoradiation has replaced surgery as first line treatment in advanced SCC of the anus, cervix, vulva and head and neck.[15–18] Chemoradiation showed excellent locoregional control rates in patients with inoperable stage III/IV head and neck SCC.[19]

In penile cancer, variable results have been observed regarding chemoradiation. Some small series comparing chemoradiation to radiation alone have shown favourable outcomes.[20–22] Two case reports revealed promising results with chemoradiation only in advanced cases.[23,24] Contrarily, two recent retrospective reviews showed more disappointing outcomes for both concurrent neoadjuvant and salvage chemoradiotherapy.[25,26] Currently, chemoradiation for both locally and regionally advanced penile cancer is prospectively evaluated at the Netherlands Cancer Institute (NCI).

Table 3: Existing reports of VRAM flap use in advanced penile cancer

Study	Year	Number of patients	Age (years)	Follow-up Period	Incidence of immediate post-op complications	Description	Comments
Santi et al.	1986	1	52	12 months	0	n/a	
Bare et al.	1994	3	Mean: 57	Mean: 5.7 months*	0	n/a	
Thomas et al.	1995	3	Mean: 61	Mean: 7 months†	0	n/a	
Brierly et al.	1998	1	48	9 months	0	n/a	
Kuntscher et al.	2006	1	44	Not reported	1 (100%)	Abdominal bulging	No mesh used at donor site
Kayes et al.	2007	4	Mean: 59	Mean: 3.5 months	1 (25%)	Flap necrosis	Required debridement
Aslim et al.	2014	5	Mean: 63	Mean: 2 years‡	1 (20%)	Donor wound edge necrosis	Required debridement and skin graft
Current series	2017	14	Median: 62	Median: 17 months	10 (71%)	See **Table 1**	

*One patient died at 5 months, another at 7 months post-operatively

†Mean of all 23 patients in series, including VRAM and anterolateral thigh flaps, not only penile SCC patients

‡One patient died at 3 months, another at 6 months post-operatively.

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Another option is induction therapy with pan-HER tyrosine kinase inhibitor targeted therapy, in which showed promising results for regionally extended disease. One-year progression free survival was comparable to the results with neoadjuvant chemotherapy in a European study, but had a somewhat lower objective response rate in comparison to neoadjuvant chemotherapy in an American study.[27–29] With a grade 3 complication rate of 11%, this therapy is well tolerated and can be considered for patients unfit to undergo combination chemotherapy.

The results in SCCs at other sites, together with the limited experience in penile carcinoma, suggest that chemoradiation should be considered in order to attempt local control in patients in whom it is unlikely that tumour-free margins can be obtained. However, management decisions remain difficult because of lack of robust data and conflicting results in the current literature.

This study suffers from the following limitations. Firstly, this is a retrospective case series with all the well-known biases. Also, this study lacks robust quality-of-life measurements, crucial for evaluating management effect in the palliative group. Thirdly, the low number of patients limited meaningful statistical analyses.

Conclusions

Despite extensive surgical resection and reconstruction with myocutaneous flaps of defects, recurrence-free survival was unsatisfying in the majority of patients with locoregionally advanced penile carcinoma. The oncological outcome was especially limited in those treated with palliative intent. Extensive surgery in this difficult patient group is associated with significant postoperative complications, and good quality-of-life measurements are lacking.

Based on these results, we suggest limiting surgery to patients in whom tumour-free margins seem achievable by all reasonable means. Likewise, for those patients in whom this goal is very unlikely, we suggest avoidance of surgery in favour of other treatment modalities, such as chemoradiation. Lastly, for future studies or case series, we suggest quality-of-life measurements in advanced penile cancer cases, as to evaluate what treatment is really adding compared to best supportive care.

Patient summary

Surgery with vascularized flap reconstruction in the treatment of large primary penis cancer tumours is associated with a considerable risk of complications,

and cure is obtained in about a quarter of patients. Therefore, we advise to limit this treatment to patients in whom tumour-free margins seem achievable by all available means.

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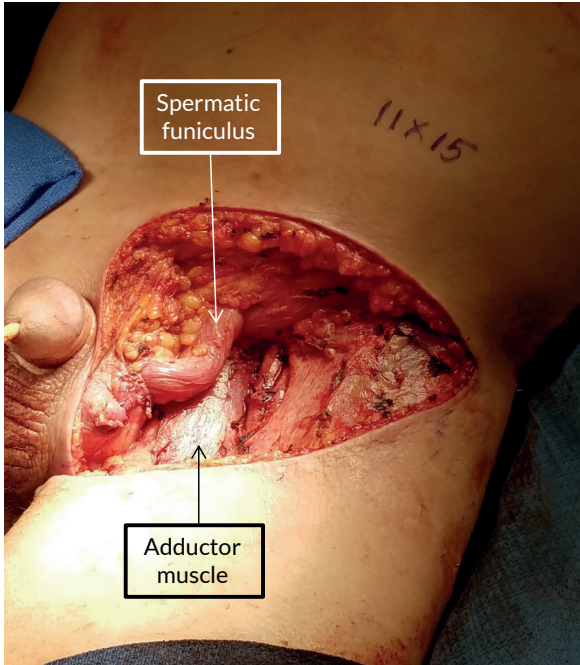
Figure S1: Inguinal Defect Measured
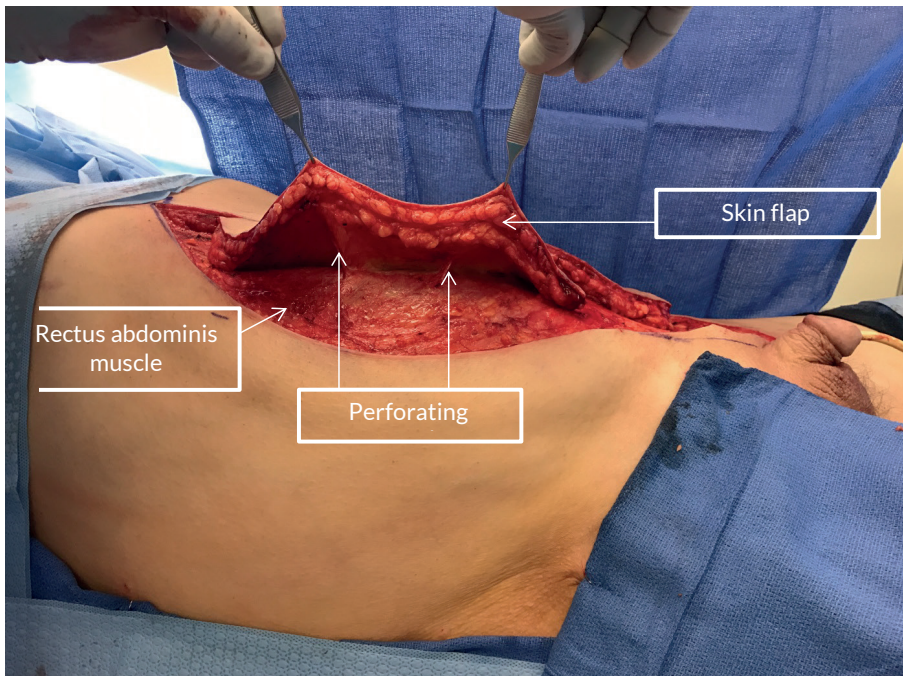
Figure S2: Demonstration of preserved blood supply to RAM flap

Chapter 8

Chemoradiotherapy as primary treatment of locoregionally advanced penile cancer

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ABSTRACT

Background: Locoregionally advanced penile cancer (LAPSCC) is associated with high treatment associated morbidity and poor survival rates, even with multimodal treatment. Chemoradiotherapy (CRT) is applied in squamous cell carcinoma from other organ sites, but its value has hardly been studied in penile cancer.

Objective: To evaluate the efficacy and toxicity of CRT for LAPSCC

Design setting and participants: We included patients fit for CRT with LAPSCC, defined as a large, with satellite lesions accompanied, or inoperable primary tumour, palpable nodes >3cm in diameter (N2/N3), suspicion of extranodal extension (ENE), or pelvic nodal involvement (N3), with no evidence of distant metastases.

Outcome Measurements and Statistical Analysis: The primary endpoint was 1-year progression free survival (PFS). Secondary endpoints were treatment response, toxicity, and overall survival. CRT consisted of 49.5 Gy in 33 daily fractions of 1.5 Gy on affected inguinal and pelvic areas, combined with mitomycin C (intravenous bolus injection 10mg/m2, maximum 15 mg) on day one and capecitabine (825 mg/m2 twice daily) on radiation days. Primary tumours and PET/CT-positive tumour deposit received a higher dose of 59.5 in daily fractions of 1.8 Gy. Responses were measured according to the PERCIST criteria for PET/CT response evaluation. Patients with incomplete responses were eligible for salvage surgery or palliative therapy.

Results and Limitations: 40 patients were included. All but one completed CRT. One-year and two-year PFS were 32%. Overall survival was 70% and 52% respectively (median follow-up of 27 months). Upon first evaluation, responses were complete (35%), partial (35%) and progressive disease (30%). At the end of follow-up, sixteen patients (40%) are alive with no evidence of disease. Sixteen patients (40%) experienced one or more therapy related severe toxicity (all grade 3) during therapy or follow-up. No therapy related grade 4 or 5 events occurred. Disease recurred or progressed in 26 patients, six had in-field failure only, six had failure outside the irradiated field only, seven had both. This study is limited by its small sample size and lack of direct comparison to treatment alternatives.

Conclusion: CRT is feasible for LAPSCC. It has acceptable survival and toxicity rates, reduces the necessity of surgical resection and can result in long-term disease free survival.

INTRODUCTION

Penile cancer is a rare malignancy. The majority of patients can be cured with surgery alone if they have no or minimal lymph node metastases.[1] However, the treatment of locoregionally advanced penile cancer (LAPSCC) remains challenging. Surgical removal only of large, multiple or pelvic lymph node metastases, as well as large primary tumours, can be burdensome and lead to disappointing disease control.[2-6] Therefore multimodal treatment is considered essential for advanced disease.[7] The most applied combination so far is neoadjuvant chemotherapy followed by surgery. Yet, even with neoadjuvant or adjuvant chemotherapy, survival rates are unsatisfactory (1-year overall survival (OS) approx. 50%) and toxicity is high (grade 3-4 events in up to 65% of patients).[8-12]

Chemoradiotherapy (CRT) plays an important role in the management of other squamous cell carcinomas such as anal carcinoma, gynaecological tumours and head and neck carcinoma.[13-15] Its role in penile cancer management has been described in smaller series and case reports, but remains largely unknown.[16,17]

In this observational study, we evaluated the therapeutic value of CRT for LAPSCC in terms of survival, toxicity and local control.

PATIENTS & METHODS

Participants
We conducted an observational cohort study in LAPSCC patients fit for CRT. Locally advanced tumour was defined as cT4, tumours with satellite lesions, tumours with a very large surface, tumours in the penile basis/bulb or tumours where radical resection was deemed unattainable. Regionally advanced tumour was defined as palpable nodes >3cm in diameter (cN2-3), fixed nodes or suspicion of extranodal extension (ENE) on imaging, or pelvic nodal involvement (cN3). LAPSCC definition included no evidence of distant metastases on baseline 18-fluorodeoxyglucose (FDG) positron emission tomography (PET) with computer tomography (CT). All patients were discussed in multidisciplinary tumour board meetings. Surgery prevailed CRT for primary tumours.

This study was approved by the institutional medical ethical committee (institutional study numbers X15PEN and N15PEN), and all participants gave written informed consent.

Chemoradiotherapy

CRT consisted of simultaneous integrated boost (SIB) intensity modulated radiotherapy (IMRT) 49.5 Gy in 33 daily fractions of 1.5 Gy on affected inguinal and pelvic areas. This was combined with mitomycin C (intravenous bolus injection 10mg/m2, maximum 15 mg) on day one and capecitabin on radiation days (825 mg/m2 twice daily, or an adjusted dose when diagnosed with DPD-deficiency). Primary tumours and PET/CT-positive tumour deposit received a higher dose (integrated boost) of 59.5 Gy in daily fractions of 1.8 Gy. Tumour deposits could shrink substantially during treatment, necessitating an adjusted radiation field in some patients.

Response Evaluation & Follow-up

Physical examination was repeated three weeks after completion of therapy. Six weeks after completion of CRT, an FDG-PET/CT was used for response evaluation, according to the PERCIST criteria for PET/CT response evaluation. Follow-up consisted of three-monthly outpatient visits to the urologist of our institute, including physical examination and an ultrasound of the inguinal nodes, with fine-needle aspiration cytology and a PET/CT scan. Deviations happened on indication and were discussed in the multidisciplinary tumour board.

Salvage Surgery & Palliative Therapy

Non-responders and partial responders with residual disease were offered salvage surgery for local disease (primary tumour) and/or inguinal and/or pelvic lymph nodes. Delayed salvage surgery was also offered to patients with locoregional recurrence or progression. If curation was not deemed possible, patients could progress with palliative therapy. Salvage surgery and palliative treatment was not considered part of study treatment.

Endpoints and Outcome Measurements

The primary endpoint was 1-year progression free survival (PFS). Secondary endpoints were 2-year PFS, overall survival (OS), local control, and serious adverse events during therapy and follow-up.

Progression free survival was measured in months from start of CRT treatment to date of progression or recurrence. A mixed response was also considered progression. Overall survival was measured from date of start treatment to date of death.

Response was measured on PET/CT imaging according to PERCIST criteria. Also, if salvage surgery was done, pathology analysis was used as a measure for response.

Toxicity, complications and other complaints were classified according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Adverse events (AE's) are grouped into therapy-related AE's, disease-related AE's and other AE's.

Therapy-related AE's are those that are regarded as (possibly) CRT related. Disease related AE's are complaints caused directly by penile tumours or metastases (e.g. tumour pain or urinary tract obstruction as a result of tumour growth). AE's classified as of other cause include complications of salvage surgery after CRT, complications of previous surgery (e.g. urostomy stenosis after partial penectomy), and other adverse events during therapy or follow-up. We excluded toxicity related to palliative therapy, and complaints in the very terminal phase can be considered incomplete as most patients were attended by their family doctor at that time.

Statistical Analysis

Median follow-up was estimated with the inversed Kaplan-Meier method. For progression free survival and overall survival, the Kaplan-Meier was used. Descriptive statistics was done with SPSS v24 (IBM SPSS Statistics, Version 24, © IBM Corp.).

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RESULTS

Between March 2014 and November 2018, we included 40 patients (characteristics summarized in **Table 1**). All patients were treated with curative intent, 29 cases with primary disease, eleven with recurrent disease.

All but one patient, were able to finish CRT. That patient had radiation therapy for a lymphoma in the past and suffered from substantial skin toxicity in the overlapping area at day ten of treatment. He stopped therapy and proceeded with palliative radiation therapy on a single painful lesion.

Seven additional patients underwent an adjusted regimen of CRT. In three patients this was due to toxicity, in four patients this was due to other reasons:

one had an inguinal hernia (lower RT dose), one received a higher RT dose (66Gy) on a primary tumour, one received an adjusted schedule (25x2Gy) on a prepubic lesion, the last patient received mitomycin instead of capecitabin because of a poor kidney function at baseline.

Table 1: Patient characteristics

Patients included		40
Median age (IQR)		64 (42-82)
WHO performance score	0/1/2	24/15/1
Setting	Primary / recurrent	29/11
cT stage*	x or 0	6
	1	3
	2	11
	3	16
	4	4
cN stage*~	x or 0	3
	1	1
	2	5
	3 (with pelvic LN)	31 (25)
Histological or cytological proof inguinal lymph node metastasis	pNx	5
	pN0	1
	pN+	34
Clinical stage	IIb	2
	IIIa	1
	IIIb	4
	IV	33
CRT field	Primary tumour region	1
	Lymph nodes	25
	Both	14

*Staging according to 2017 TNM classification. ~At physical examination or imaging.

Response, Locoregional control and recurrences

Upon first evaluation after therapy (mean 6.7 weeks after therapy), fourteen patients (35%) had a complete response, another fourteen patients (35%) had a partial response, and twelve patients (30%) showed progression (the latter including four patients with mixed response). Twenty-five patients (63%) achieved a complete remission of disease during the study period. However, only sixteen of them (40% of total cohort) were disease free at the end of follow-up. Responses and endpoints are listed in **Table 2**, disease course of all patients is graphically depicted in **Figure 1**.

Figure 1: Plotted disease course and treatments. Each bar represents one patient.
DD; Dead of disease, AWD; alive with disease, NED; no evidence of disease.

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Fourteen patients remained free of recurrence or progression during follow-up, half of them with initial complete response, the other half with initial partial response. Disease recurred or progressed (so-called treatment failures) in 26 patients, seven of whom had multiple failures during the study period. Thirteen of 26 patients had in-field failures (50%), four with initial complete response, four with initial partial response, five with failure upon first evaluation. Of six patients with out-field failure only, just one initially responded to CRT (partial responder). Of seven patients that failed both in and out of the radiated fields, three had initially responded completely to CRT, two initially had a partial response.

Salvage Surgery & Palliative Therapy

A schematic overview of additional treatments such as salvage surgery and palliative therapies is provided in **Figure 2.** In total, nineteen patients underwent either immediate (after first response evaluation, n=11), delayed (median 8.0 months, n=7) or palliative (n=1) surgical resection. Surgery included the primary tumour (n=6), lymph nodes (n=10), or both (n=3). Immediate surgery was done at a median of 5.4 months after start of chemoradiation therapy (range 2.8-5.6). Delayed surgery was done at a median of 8.0 months after start of chemoradiation therapy (range 4.5-11.3). The indication for additional surgery was: recurrence, progression, or persistent disease defined as ambiguity or residual metabolic activity. One resection was done only for palliative reasons.

Of fourteen complete responders, four patients (29%) underwent immediate or delayed salvage surgery. Of fourteen partial responders, eleven underwent salvage surgery (79%). Palliative treatment was given to nine patients.

Survival

Median follow-up was 27 months (95%CI[20-35]). All deaths were penile cancer related. PFS was 32% at both 1 and 2 years (**Figure 3**). Median time to progression was 6.9 months (95%CI[3.7-10.1]). Progression/recurrence during the second year of follow-up did occur only in patients who previously had progression, recurrence or a mixed response in the first year. In other words, all patients that remained free of progression or recurrence in the first year (n=11), remained progression-free for the rest of their follow-up.

Of the entire cohort, 1- and 2-year OS was 70% and 52% respectively (**Figure 4**). Median OS was 25.3 months (95%CI[9.7-41.0]). Of those with progression, 1- and 2-year OS was 55% and 31% respectively (median just over one year but with a very wide confidence interval (95%CI[6.6-20.9]).
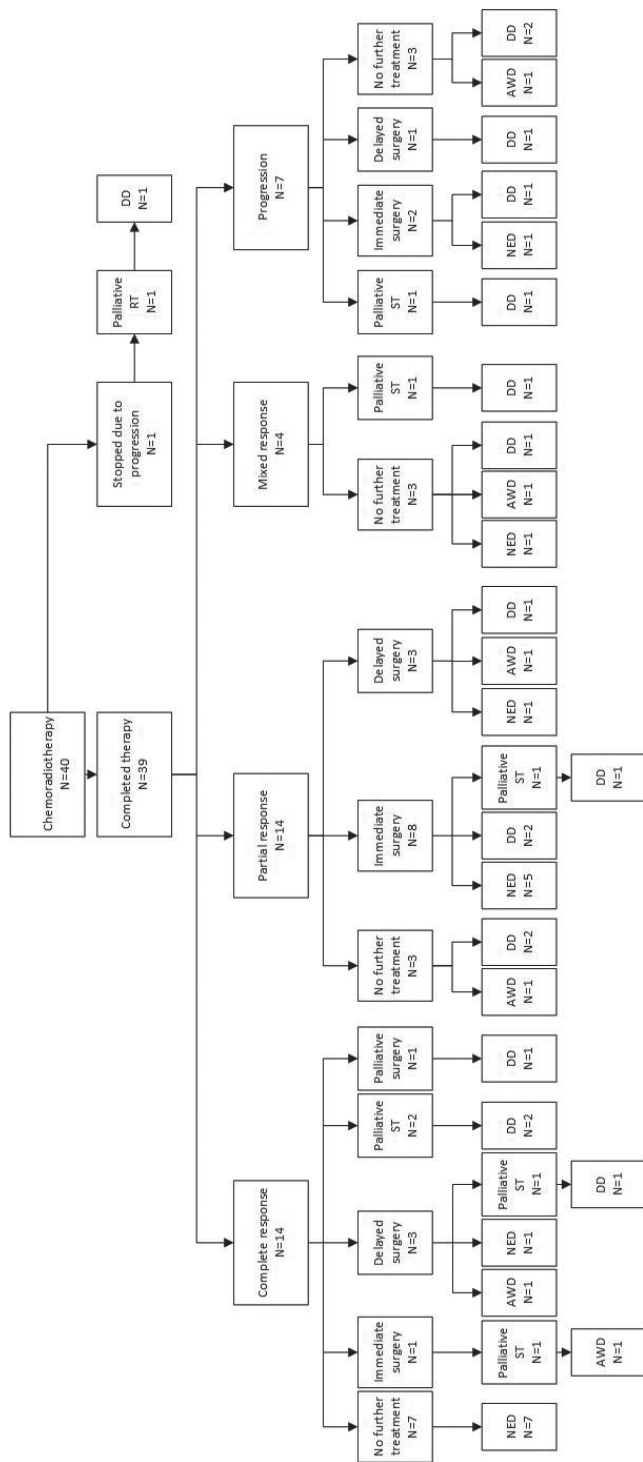
Figure 2: Flowchart of treatments and patient status.
ST; systemic therapy, RT; radiotherapy, NED; no evidence of disease, AWD; alive with disease, DD; dead of disease.

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Table 2: Response to treatment, failures and patient status

Initial response	N	Best response*	N	Failures N (multiple*)		NED	AWD	DD
Complete	14	Complete	14	In-field	4 (2)	8	2	4
				Both	3 (1)			
Partial	14	Complete	7	In-field	1	6	1	0
		Partial	7	In-field	3	0	1	6
				Out-field	1 (1)			
				Both	2			
Progression	12	Complete	4	In-field	4 (2)	2	0	2
		Partial	1	Out-field	1	0	1	0
		Progression	7	In-field	1	0	1	6
				Out-field	4			
				Both	2			
Total	40		40		26	16	6	18

Responses according to PERCIST criteria. * Response to CRT or remission achieved during study period. NED; no evidence of disease, AWD; alive with disease, DD; dead of disease.
*later failures include in-field failure, out-field failure and/or both.

Toxicity

All toxicities and surgical complications are described in **Table 3**. No therapy related deaths occurred. One patient (2,5%) discontinued therapy because of toxicity in the radiated field (described under participants). Three patients (7,5%) stopped capecitabin early or continued with a lower dose. One of them received an extra course of intravenous mitomycin.

In total, 114 events were registered: 72 grade 2, 39 grade 3, and 3 grade 4 events. Thereof, respectively 50 grade 2 and 22 grade 3 events were (possibly) therapy related. All three grade 4 events were septicaemia, two wound infections after ILND, and one exceptional case of an abdominal sepsis due to intestinal metastatic lesions causing faecal leakage.

Seven patients (18%) finished therapy and follow-up with only mild therapy related symptoms (grade 1 toxicity), five of them who also did not experience disease related or other symptoms.

Sixteen of 40 patients (40%) experienced one or more (possibly) therapy related grade 3 toxicity during therapy or follow-up. The majority (11/16, 69%) with onset during therapy, two with onset before therapy (tumour pain and a

skin infection) and four with onset during follow-up (worsening skin problems, anaemia and lymphedema).

Seventeen surgery-related events were registered in 9 patients that underwent lymph node surgery. Lymphedema was mostly due to lymph node dissection, only two patients had lymphedema due to the study treatment (three events, as one later progressed from grade 2 to grade 3).

Overall, toxicities were transient. Of all 114 registered toxicities and complications, 20 (18%) resolved within a week, an additional 56 events (49%) resolved within one month. Only four events (3,5%, all lymphedema) lasted longer than a year.
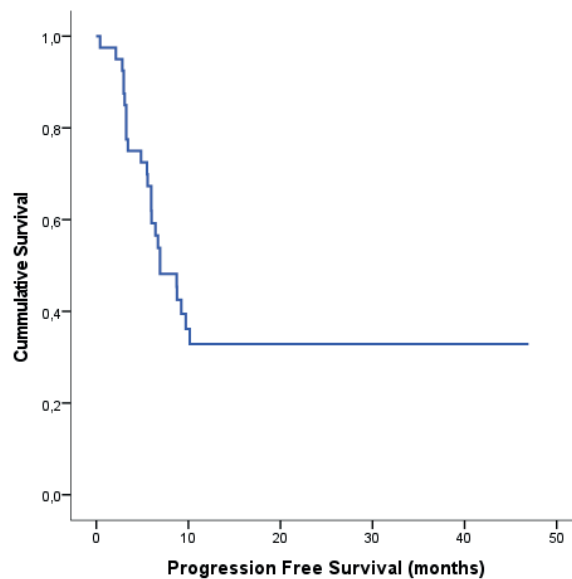
Figure 3: Progression free survival according to Kaplan-Meier estimates

Time interval	1 yr	2 yr	3 yr	4 yr	5 yr
No at risk	40	11	7	5	0
Events	26	0	0	0	-
Cum. PFS	32%	32%	32%	32%	-
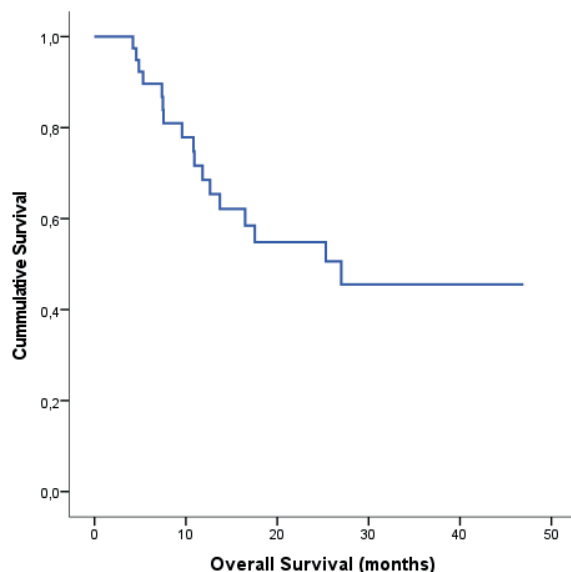
Figure 4: Overall survival according to Kaplan-Meier estimates

Time interval	1 yr	2 yr	3 yr	4 yr	5 yr
No at risk	40	22	13	6	0
Events	11	5	2	0	-
Cum. OS	70%	52%	42%	42%	-

Histopathological findings after surgery

No vital tumour cells were found in resected tissue of six patients that were suspected of tumour deposits. In four of these patients this regarded the groin where surgery was preceded by tumour-positive fine needle aspiration cytology (FNAC) 5-8 weeks earlier. For the other two, PET/CT showed a partial response (of the primary tumour in one patient, of the groins in the other). None of these six patients had a disease recurrence during follow up.

Vital tumour cells were found in thirteen cases; in seven patients after immediate surgery, in five patients after delayed surgery, in one after palliative resection. After immediate resection of vital tumour deposits, two patients remained free of disease recurrence. After delayed surgery and finding vital tumour deposits, only one patient remains free of recurrence (but had no follow-up imaging after surgery yet, classified alive with disease).

Fifteen patients were treated with CRT on their primary tumour lesions. Nine of them (9/15=60%) received either immediate (n=4) or delayed (n=5) surgical resection. Histopathological evaluation showed no vital tumour cells in four, and vital tumour residue in five patients. No relation was found between timing of surgery and pathological results.

Table 3: Toxicities and surgical complications
UTI; urine tract infection.

	Grade 2			Grade 3/4		
Skin	**Therapy**	**Disease**	**Unrelated**	**Therapy**	**Disease**	**Unrelated**
Radiation dermatitis	**19**	-	-	**9**	-	-
Other skin problems	**1**	-	-	**2**	-	-
Gastro-intestinal						
Diarrhoea/radiation enteritis/cystitis	**4**	-	-	**1**	-	-
Nausea/anorexia/vomiting	**2**	-	-	**-**	-	1
Weight loss	**2**	-	-	**-**	-	-
Dysphagia	**-**	-	-	**-**	-	1
Local						
Pain	**8**	2	1	**1**	1	-
Urethral/meatal obstruction	**3**	2	1	**-**	1	-
Urinary fistula	**-**	1	-	**-**	-	-
Genital infections	**-**	1	-	**-**	1	-
General						
Anaemia	**2**	-	-	**1**	-	-
Electrolytes/other laboratory findings	**4**	1	-	**2**	-	-
Collapse	**-**	-	-	**1**	-	1
Thrombo-embolic event	**-**	-	-	**-**	1	-
UTI	**-**	-	1	**-**	-	-
Infections (other than local or wound)	**-**	-	2	**2**	1	2
Fatigue/dyspnoea	**3**	1	-	**1**	-	-
Fever e.c.i.	**-**	-	1	**-**	-	-
Malaise	**-**	-	-	**1**	-	-
Groins and wounds						
Inguinal wound infections/seroma	**-**	-	4	**-**	1 (sepsis)	5 (2 sepsis)
Wound dehiscence	**-**	-	1	**-**	-	-
Lymphedema	**2**	-	3	**1**	-	4
Total	**50**	8	14	**22**	6	14
Total per grade		72 grade 2			42 grade 3/4	

The same was found for salvage surgery of the groins. Thirty-nine patients underwent CRT of one or both groins, thirteen underwent immediate (n=8) or delayed (n=5) lymphadenectomy. Six with no vital tumour, seven with vital tumour residue, and no relation between timing of surgery and pathological results.

DISCUSSION

This study shows that CRT (mitomycin, six weeks of capecitabin and radiation) is a well-tolerated treatment for patients with advanced stages of disease. We found 1- and 2-year PFS of 32%. Overall survival was 70% and 52% respectively (median follow-up of 27 months). These survival rates are comparable to those of other multimodal approaches. Despite a toxicity rate of 40% (grade 3 therapy related events), CRT appeared less toxic than neoadjuvant chemotherapy because complaints were mostly transient and therapy could be continued. These study results mean that CRT is a treatment alternative to neoadjuvant chemotherapy or adjuvant radiation with surgical resection.

In studies with neoadjuvant chemotherapy, some find lower toxicity rates, but high rates are not unusual.[4,10,12,18,19] In our experience with neoadjuvant chemotherapy (TPF) for locoregionally advanced disease, 23% of the patients had to stop therapy due to toxicity, an additional 30% because of disease progression. This contrasts with only one patient discontinuing in the current study. Furthermore, in just over half of the patients in current study surgery was avoided.

In terms of progression free and overall survival current results resemble previous findings, although response rates vary in previous studies.[8,10,12,20] In aforementioned TPF cohort 1-year PFS was 31%, (32% in the current cohort), and further decreased to 12% after 2 years, while in current cohort the remainder of patients stayed free of recurrence.[8] OS seems better in current cohort, 70% vs. 46% in the first year of follow up, and 52% vs. 27% in the second year. This may be partly explained by including lower stages of disease, as current study also included bulky T3 tumours and large inguinal lymph nodes (cN2) instead of only T4 and N3 disease.

A study by Kent et al. described chemoradiation in a cohort (n=26) of men with primary urethral carcinomas. One year disease free survival was 61%, 1-year disease specific survival was 83%. Their higher rates of disease control and survival is probably due to earlier disease stages in their cohort: only nine

patients had nodal disease (N2 at worst).[21] Their results also suggest that trials with chemoradiation in lower stages of penile cancer may be of value.

A Danish study cohort recently described CRT adjuvant to ILND for inguinal lymph nodes with extranodal extension (N3). In only one of eleven patients PLND was performed. The 5-year penile cancer survival probability was 57%, which is comparable to survival rates described by Li et al for the N3 patients without positive pelvic lymph nodes.[22,23] The Danish penile cancer study group shows that CRT can be used as curative therapy instead of a prophylactic PLND. Whether this reduces toxicity remains unestablished. Neither the population or the setting of CRT is comparable to our current study.

In this study we were unable to identify the optimal timing of salvage surgery, the optimal timing of response measurement, or the reliability of response measurements. Based on few cases in our study, CRT may not be feasible for previously irradiated areas and fine needle aspiration biopsy may not be reliable in discriminating PET-positive nodes with and without residual disease. The cases where FNAC showed tumour cells but surgery did not suggest that a treatment effect is ongoing in the weeks between. Also, the role of CRT in pain control is insufficiently evaluated in this cohort. A few patients had remarkably less pain during treatment then before, however, some patients had pain due to tumour necrosis or tumour growth.

Showing that CRT is an alternative treatment is important because treatment of advanced stages is burdensome and despite treatment associated with rapid disease course. Optimizing treatment combinations in a multimodal approach is essential in order to improve patient outcomes. In comparison to perioperative chemotherapy studies, the omission of surgery is a potential advantage of CRT, with only half of our patients proceeding to surgery, compared to over 75% with perioperative chemotherapy.[8,10]

Our findings are subject to several limitations. This is a small observational cohort study, despite being the largest so far, with inherent bias due to the design. As penile cancer is a rare disease (in total n=150 per year in the Netherlands), a randomized trial was not feasible.[24] Therefore, only indirect comparisons to treatment alternatives such as neoadjuvant chemotherapy can be made.

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Secondly, no quality of life (QOL) data in the form of patient reported outcome measures (PROMs) were collected. With the ultimate goal not only to improve survival but also patient's QOL, we feel the lack of PROMs as a serious omission.

Lastly, we did not determine human papilloma virus (HPV) positivity in tumour samples of our subjects. HPV+ penile tumours have better disease outcomes, and a better response to CRT is shown for HPV+ tumours on other sites.[25,26]

Strengths of this study include a single centre design where all treatment decisions were made by an experienced multidisciplinary team, and the novelty of primary CRT as a treatment approach for penile cancer. Further follow up of this cohort will provide more insights in response endurance and survival. More experience with CRT and other (multicentre) studies will also aid treatment optimization.

Conclusions
Chemoradiotherapy is feasible as a treatment for advanced non-metastatic penile cancer, with a median OS of 23 months, and an enduring remission in >25% of patients. Associated toxicity rate is acceptable (40%). Although salvage surgery following CRT is feasible, it can be avoided in 50% of patients.

Acknowledgements

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Chapter 9

General discussion and future prospects

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GENERAL DISCUSSION AND FUTURE PROSPECTS

Penile cancer is a rare but potentially mutilating and lethal disease. Disease management has evolved towards penile sparing surgery for primary tumours and minimally invasive staging of lymph node metastases (sentinel node biopsy). The majority of patients have no metastases at presentation, a smaller part (10-20%) presents with lymph node metastases (LNM) or worse. Curing metastatic disease can be challenging, especially with LNM and extranodal extension or with pelvic LNM.

In this thesis we focussed on the advanced stages of penile cancer: we found targets for new therapies, we described early detection of metastases, and we evaluated the current surgical treatment and an experimental treatment for advanced disease.

Immunology and immunotherapy

The patients' immune system plays a critical role in various tumour types, including squamous cell carcinomas. Immunomodulatory mechanisms exploited by malignant cells aim to cripple or bypass anti-tumour immunity. One of these mechanisms is targeting key signalling events for T-cell responses such as co-inhibitory or co-stimulatory molecules, also known as check-points. Although the introduction of checkpoint inhibitors has revolutionized the treatment of advanced stages of cancer, only a small percentage of patients responds to this treatment and biomarkers to predict response to immunotherapy are currently an active field of investigation. Even well-studied checkpoint markers such as PD-L1 and PD-1 are imperfect selectors for immunotherapy.[1–4]

Different immune escape mechanisms have been described in penile cancer, amongst other in **chapter 2** and **chapter 3**.[5–10] The results presented in this thesis show that primary penile tumours use different immune escape mechanisms, including PD-L1, non-classical HLA, regulatory T-cells and immunemodulating macrophages. **Chapter 2** describes that almost half (48%) of penile tumours express PD-L1. Of the PD-L1 positive tumours, a pattern of expression mainly at the tumour margin is associated with a better survival while a diffuse expression pattern is associated with worse survival. In **chapter 3** PD-L1 expression is analysed together with hrHPV status, HLA class I expression, presence of regulatory and cytotoxic T-cells and presence of tumour infiltrating myeloid cells. In univariable analysis, unfavourable clinical outcomes were associated with tumour PD-L1 expression, negative hrHPV status, presence of CD163+ tumour

infiltrating macrophages, non-classical HLA class I upregulation and low stromal CD8^{+} T-cell infiltration. In multivariable analysis, PD-L1 expression and HPV-status remained significant predictors of outcome. Although this is the most comprehensive immunohistochemical study so far and the results favour trials of immunotherapy in penile cancer treatment, the complexity and dynamic immune cell-tumour interplay of the TME is far from unravelled. We have not analysed activation status of T-cells, nor expression of checkpoint molecules on T-cells (e.g., PD-1, CTLA-4, TIM3, LAG3) on tumour cells, we have not performed proximity analysis, nor have we studied soluble immunemodulating factors (e.g. IFNγ), etcetera. Moreover, both studies regard primary tumours, and most patients die from metastases.

Unpublished work from our group (manuscript in preparation), focused on a comprehensive analysis of the various T-cell subsets present in HPV[+] and HPV[−] primary penile tumours and tumour draining lymph nodes. Multicolour flow-cytometry was performed on fresh material from resected inguinal lymph nodes of penile cancer patients in a similar manner as previously published for cervical cancer patients.[11] Notably higher rates of activated and proliferating T-cells (and co-inhibitory marker positive T-cells (CTLA-4, PD-1) were seen in HPV[+] tumours compared to HPV[−] tumours. This suggests that HPV[+] tumours are more immunogenic because of their viral causation. In tumour positive lymph nodes, there were significantly higher rates of regulatory T-cells, and more expression of the checkpoint molecules CTLA-4/PD-1 and LAG3 checkpoint molecules on various T-cell subpopulations. This indicates that combinational immunotherapy targeting CTLA-4 and PD-1 might be beneficial in metastatic penile cancer. This has been given in early phase trials with promising results warranting further analyses.[12,13]

Of course, immune response and escape mechanisms are dynamic processes that cannot be fully studied with resected specimen immunohistochemistry alone. Besides aforementioned additional markers and factors, it would be interesting to look further into different mechanisms of PD-L1 expression as discussed in **chapter 2** and compare these data with genetic profiles of the primary tumour. The study on the microenvironment of tumour-draining lymph nodes will improve with more samples and validation. Also tissue imprints from bisected lymph nodes can be used for proximity analysis and pattern recognition (like in **chapter 2** a marginal PD-L1 expression pattern was recognised). For the present and near future it is crucial to have close immune-monitoring during immunotherapy trials, including at least typing of tumour cells, T-cells and myeloid cells (including activation and/

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or exhaustion markers, proliferation status and checkpoint expression), possibly complemented with analysis of tumour-derived factors (interleukins, interferons, growth factors). Analyses are preferably done before, during and after therapy as this would provide insights in the immunological changes in the TME with therapy, and mechanisms exploited in and around tumour residues after therapy.

The ample presence of i.a. Tregs and PD-L1 expression provide a rationale for immunotherapy trials (**chapters 2 and 3**). However, the clinical value and effect of immune modulating agents in penile cancer remains largely unknown as hardly any clinical trial has finished yet. Based on the results of **chapter 2 and 3**, the PERICLES clinical trial started in the Netherlands Cancer Institute and is still running (checkpoint inhibitor anti PD-L1 with or without radiotherapy; EudraCT-number: 2018-000603-17). Trials targeting CTLA-4 (Tregs) or combination trials that target both CTLA-4 and PD-1/PD-L1 also seem rational as we found that these checkpoints were expressed in our studies (manuscript in preparation). Hopefully, immunotherapy can be of value to those with distant metastases where cure is now impossible. Importantly, immunotherapy dramatically changed the prognosis of stage 3 melanoma and metastasized renal cell carcinoma.[14,15] Whether immunotherapy can improve disease control in patients with multiple lymph node metastases where surgery alone is not sufficient, also needs further investigation.

The human papilloma virus (HPV) is associated with 25-50% of invasive penile cancers, with oncogenic HPV type 16 being the most prevalent.[16-18] HPV-positive (HPV+) tumours generally have a better prognosis than HPV-negative (HPV-) tumours, even the metastasised tumours.[18] A tendency to respond better to treatment is presumably contributing to this survival benefit.[19,20]

Vaccination against HPV, reduces the incidence of HPV-associated lesions and was included in the Dutch national immunisation programme years ago, for girls only.[21] In June 2019, the Health Council of the Netherlands advised vaccination for boys as well.[22] From 2021 boys will be offered the vaccine at 9 years of age.[23] This is expected to reduce the incidence of penile and other HPV-induced cancers in men and women in the future.

Beyond prevention, there may also be a therapeutic role for HPV vaccination for penile lesions. Studies in cervical intraepithelial neoplasia (CIN, virtually always HPV+) show a lower recurrence rate after conisation and vaccination as compared to the control group that underwent conisation alone, as reviewed by Jentschke

et al.[24]. In men, HPV vaccination was moderately effective against persistent anogenital HPV infection (47% efficacy) and anal intraepithelial neoplasia (47-62%).[25] These studies support the expectation that adjuvant vaccination will be of value in PeIN as well, but this still needs scientific evaluation.

Although outside the scope of this thesis, the association between HPV status and patient outcomes in penile cancer is in part mediated by epigenetic effects of viral oncogenes or mutational status of the patient. Possibly, expression of immunogenic viral oncogenes is a favourable factor in HPV$^+$ tumours, contributing to a better tumour recognition and T-cell reactivity. Studies on mutational burden in penile cancer show a relatively low mutational burden (3.6-4.4 mutations/Mb) compared to e.g. melanoma and bladder cancer that both respond well to immunotherapy.[26,27] A high mutational burden results in a more diverse neoantigen repertoire that can be presented to cytotoxic T-cells, that - after removal of the supressing checkpoint mechanisms - results in a strong anti-tumour response.[28] Following this line of thought, the low mutational burden of penile cancer may suggest a moderate response to checkpoint inhibitor therapy. However, clear cell renal cancer has an even lower mutational burden (around 1.1 mutations/Mb), while immunotherapy has substantially improved its prognosis.[15,29,30]

In various squamous cell cancers including penile cancers, *TP53* is associated with HPV$^-$ tumours and with inferior survival.[26,31,32] *PIK3CA* mutations were more often seen in HPV$^+$ anal and oral SCC than in HPV$^-$ tumours.[31,33] *PIK3CA* mutations have also been described as mostly associated with early stages (PeIN) of penile malignancies, not with poor survival or LNM.[34,35]

Neither covered in this thesis is targeted therapy for penile cancer. The epithelial growth factor receptor (EGFR) is frequently expressed in penile cancers, and a study by Necchi et al showed that targeting EGFR signalling with anti-tyrosine kinase therapy is safe and effective.[36,37] Possibly, targeted therapies can be used when chemotherapy is not an option. Also, combination therapies can be further explored.[38]

Imaging and staging
Currently in penile cancer, FDG-PET/CT imaging is not used to its full potential in advanced stages of the disease (**chapters 4, 5 and 6**). In the nearby future, it may be used for earlier and non-invasive diagnosis of tumour-positive pelvic lymph nodes, and early discovery of distant metastases, as suggested in this thesis. Also, one study described a possibly relevant role in clinically node-negative patients

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when used with the sentinel node biopsy.[39,40] Note that small metastases can be missed with all imaging modalities, and that pathologic staging prevails for definitely ruling out metastases.[41]

The role of PET/CT for response evaluation after systemic or radiation therapy should be evaluated for penile cancer.[42] Currently this is hardly described in literature, while in this thesis (**chapter 8**), FDG-PET/CT was used to measure response.

Ideally, PET/CT will become more specific for penile cancer, comparable to the successful PSMA-PET/CT for prostate cancer.[43] In other cancers different PET tracers are studied, for example markers of proliferation, hypoxia and angiogenesis.[44,45] Such detection methods possibly improve test performances of PET/CT and hopefully lead to a better diagnosis and monitoring. Yet, these prospects are still far away in penile cancer staging. In lung cancer, PD-L1 has been labelled for PET/CT.[46] Although it has not been correlated to checkpoint inhibition response, it could be a way to non-invasively quantify PD-L1 expression in patients.

Staging and treatment of penile cancer involves more factors than the current TNM classification includes. Since 2020, the EAU guidelines recommend assessment of HPV-status of resected tumour specimens as a prognosticator. A more precise clinical staging system including HPV-status will lead to better risk stratification. Another example is a subclassification of N3 that was proposed by Li et al.[47] They showed that dividing the inguinal metastases with extranodal extension (ENE; N3a) from the pelvic metastases (N3b) better reflects the prognosis of these subgroups.

So far, however, HPV-status does not impact disease management.[48] Besides a better prognosis, studies also suggest a better response to treatment for HPV⁺ tumours.[18,20,49] Therefore the treatment of HPV⁺ tumours may become less aggressive than the treatment of HPV⁻ tumours in the future. This has already been suggested for chemoradiotherapy in oropharyngeal tumours.[50,51]

Although risk stratification with (sub)staging is useful, the real importance is that staging and treatment is adequate and optimal. This is best reached by timely referral to a specialized centre.

Literature shows that penile cancer care is best in specialized centres. Williams et al reviewed the impact of centralization in genitourinary cancer and found better utilization of surgery, lower morbidity and better survival outcomes in high-volume centres, especially for major surgical procedures.[52] For penile cancer specifically, an American study showed that patients treated in non-academic centres were less likely to undergo invasive lymph node staging when indicated, while this notoriously results in a higher risk of mortality.[53,54] Furthermore, centralization also improves histopathological diagnostic accuracy and adherence to guidelines.[55,56]

In penile cancer therapy, there is a pressing need to decrease the morbidity associated with inguinal lymph node dissections. There are at least three approaches: 1) improvements of surgical techniques, 2) alternative staging methods and 3) alternative treatments.

1. Various modifications of ILND techniques have been reported to lead to lower complication rates. Endoscopic ILND showed promising results in various studies[57] and efforts are made in wound treatment after ILND.[58] However, patient numbers are small and complication rates remain high. Also, a reporting bias is may be present as it regards complications.

2. The most effective strategy to prevent complications from ILND is to avoid ILND completely. This goal has been reached by introducing the sentinel node biopsy procedure and modified ILND instead of staging radical ILNDs. Nevertheless, a tumour-positive SN procedure is always followed by ILND. In stage III melanoma patients, inguinal sentinel node procedure can safely be followed by close follow-up. An immediate completion lymph node dissection was shown to improve regional control, but not melanoma specific survival.[59,60] Whether this also applies to penile cancer is unknown. Currently a European collaboration study investigates the factors predicting metastatic non-sentinel lymph nodes. Preliminary results suggest a relation between size of the metastasis in the sentinel node and additional metastases in the completion ILND.[61] Replacing a staging ILND with a minimal invasive procedure requires careful investigation, as delayed resection of occult metastases can be fatal.[54] In 2007, for vulvar cancer a suggestion was made to replace a staging inguinal lymphadenectomy at the time of primary tumour resection with radiotherapy. The authors concluded that radiation was optional for early-stage vulvar cancer, and that prospective studies were warranted.[62] Later, a review described lower morbidity of radiation, but a possibly higher risk of recurrence.[63] With a limited quality of studies it is difficult to determine exactly the potential of radiotherapy.

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3. Therapeutic ILND can also be avoided with treatment alternatives. Better non-invasive staging (imaging) before ILND can reveal that multimodal therapy is indicated (**chapters 6 and 8**). And in cases of complete response, or at worst, progression to incurable disease, therapeutic lymph node dissections may be avoided. Between 2000 and 2014, 13% of all patients that underwent pathologic staging at the NCI were staged pN3 (unpublished data). These patients account for 40% of therapeutic ILNDs and would ideally be identified beforehand.

Therapy for advanced stages of disease

More surgery is not always successful. The results in **chapter 7** of this thesis describe a small cohort (n=15). Nevertheless, these data support previous reports where experts describe that major surgery should only be performed when carefully considered.[64] Given the poor oncological and surgical outcomes, such major surgery should be mainly used for palliative reasons such as tumour pain and (other) daily care problems. Its use in curative setting will be mostly in a combination of multiple therapies, or after carefully considering its benefits and risks.[64,65]

For the treatment of the inguinal lymph nodes significant differences exists, even between neighbouring countries. Treatment strategies range from ILND with adjuvant radiation therapy to ILND with neoadjuvant and adjuvant chemotherapy. It would be of great value to compare experiences in treatment strategies between centres in terms of efficacy, toxicity and complications, recurrences and also quality of life. This would be possible within an international collaboration. A project of that nature is the InPACT trial that prospectively evaluates benefits and sequencing of surgery, chemotherapy and chemoradiation therapy.[66] Results are eagerly awaited.

Despite advancements in treatment of lymph node metastasis, survival of advanced stages of the disease is low. Clinically N3 patients have a 5 year survival of 20-35%.[65,67,68] Large primary lesions do not fare well either as described in **chapter 7**. According to guidelines multimodal treatment is indicated in these advanced stages. Neoadjuvant chemotherapy is the most studied option so far with platinum based regimens now considered the most efficacious, and taxane based regimens the most toxic.[69]

Although radiotherapy is not recommended in current EAU guidelines, we saw good radiosensitivity in the chemoradiation trial (**chapter 8**).[48,70] Also, radiotherapy has been used as adjuvant to lymph node surgery in our centre

for years, showing survival benefit in a four-centre report.[71] On the other hand, some patients' disease progressed during chemoradiation therapy, some even had disease progression within the radiated fields. What is needed is a marker of sensitivity for radiotherapy. Yuan et al studied radiosensitivity of penile tumours and advocate dose prescription based on the individual tumour biology, as radioresistance was mostly present but varied significantly.[72]

HPV-status could be one of these markers as better response was seen in HPV+ head and neck cancer and has also been suggested in penile cancer.[19,20,73,74] Some studies investigated oxygen levels within tumours to predict response to radiation.[74,75] Also injection of immunotherapeutic agents within tumours masses has been studied, showing response in both injected and other lesions in melanoma.[76-78] Results in cervical carcinoma are awaited.[79]

But even if all best reported results of current options were achieved in all hospitals, a problem remains with therapy resistant, large, inoperable and widely spread disease. Whether it consists of combinations of current options, targeted therapies, immune-modulating treatments or best supportive care: more options need to be explored and compared with each other in order to find solace for these patients with a poor prognosis. Considering quality of life and other patient reported outcome measures in such studies is crucial.

The major flaw of the last chapters of this thesis (**chapter 7 and 8**) is the lack of quality of life data. Although patient reported outcome measures (PROMS) may not provide all information (e.g. coping strategies, expectations) it is essential information in the management of such disease. Patients may experience advantages and disadvantages of the treatment options differently than we think. Quality of life data will definitely contribute to making the best treatment decisions with the patient.

In conclusion, in the present thesis we point to possible immunotherapy for penile cancer targeting the PD-L1 and/or CTLA-4 checkpoints. We advocate earlier detection of pelvic lymph nodes and distant metastases with better use of FDG-PET/CT, careful consideration of large surgical procedures, and present chemoradiation as a multimodal treatment alternative for advanced stages of penile cancer.

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Chapter 10

Summary / Samenvatting

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SUMMARY

Penile cancer is introduced in **chapter 1**, describing in short the epidemiology, aetiology and risk factors, tumour-immunology, imaging and treatment of the disease.

Chapter 2 and 3 cover immunological aspects of penile cancer. We compared expression of various immune factors with disease specific survival (DSS) and lymph node metastases (LNM) at diagnosis. Therefore we used archived tumour tissue. The role of programmed death ligand-1 (PD-L1) was studied in **chapter 2**. PD-L1 is an immune checkpoint molecule that can be expressed by tumour cells or immune cells. It inhibits T-cell function when bound to its receptor. Anti PD-L1/PD-1 immunotherapy can block its function and enhance tumour eradication by T-cells. We looked at PD-L1 expression in different areas of the tumour microenvironment (TME): tumour, stroma, and tumour-infiltrating macrophages. Also, the pattern of expression was noted, that is diffuse or predominantly present at the tumour-stroma margin.

Of 200 tumours 96 (48%) were PD-L1 positive (scored 1% or greater). Of PD-L1$^+$ tumours, 59 (62%) had a marginal expression pattern and 79 (82%) were high risk human papilloma virus (hrHPV) negative, compared to 75% in the whole cohort (p = 0.03). Compared to PD-L1 negative tumours, the PD-L1 expression patterns had different prognostic values in the whole cohort as well as in the high risk HPV negative subgroup. On multivariable analyses a marginal expression pattern was associated with absent lymph node metastases (odds ratio (OR) 0.4) while diffuse expression was associated with poor survival (hazard ratio (HR) 2.58). These results were more prominent in the high risk HPV negative subgroup (OR 0.25, HR 3.92).

In **chapter 3**, more immune factors of the tumour microenvironment are studied. Analysis included previously described hrHPV status, immunohistochemical scores for classical and non-classical human leukocyte antigen (HLA) class I, PD-L1, and novel data on tumour-infiltrating macrophages and cytotoxic and regulatory T-cells. Clinicopathological characteristics and extended follow-up were also included.

In the total cohort (n = 216), diffuse PD-L1 tumour-cell expression, CD163$^+$ macrophage infiltration, non-classical HLA class I upregulation, and low stromal CD8$^+$ T-cell infiltration were all associated with LNM. In the multivariable model,

only tumour PD-L1 expression remained a significant predictor for LNM (OR 2.8, p = 0.05). hrHPV negativity and diffuse PD-L1 tumour-cell expression were significantly associated with poor DSS and remained so upon correction for clinical parameters (HR 9.7, p < 0.01 and HR 2.8, p = 0.03). The only immune factor with different expression in HPV+ and HPV− tumours was PD-L1, with higher PD-L1 expression in the latter (p = 0.03).

In the HPV− cohort (n = 158), LNM were associated with diffuse PD-L1 tumour-cell expression, high intra-tumoral CD163+ macrophage infiltration, and low number of stromal CD8+ T-cells. The first two parameters were also linked to DSS. In the multivariable regression model, diffuse PD-L1 expression remained significantly unfavourable for DSS (HR 5.0, p < 0.01).

These results emphasize the complexity of the tumour microenvironment in penile cancer and point toward several possible immunotherapy targets. Here described immune factors can aid risk-stratification and should be evaluated in clinical immunotherapy studies to ultimately lead to patient tailored treatment.

Chapter 4 reviews staging and imaging of penile cancer. According to the literature reviewed, T-staging relies on physical examination, but MRI can aid a more sensitive staging if needed. N-staging is most complicated for small metastases, as they are prone to be missed. For the detection of small metastases, the (dynamic) sentinel node biopsy (SNB) procedure is preferred because it provides accurate pathological staging without the side effects and complications of an inguinal lymph node dissection (ILND). SNB should be preceded by US-guided FNAC to avoid undetected macrometastases. FNAC also is a valuable tool to confirm metastases in suspicious groins. Nevertheless, micrometastases can be missed with every non-invasive or minimally invasive staging tool. Additional efforts should be made to further reduce false-negative rates.

Highly suspicious groins require inguinal lymph node dissection as it is of great therapeutic value. For M-staging, conventional anatomical imaging is recommended. High sensitivity has been described for FDG-PET/CT. Its role was further evaluated in **chapter 5 and 6** for both N and M-staging. Follow-up and surveillance rely on physical examination and US-FNAC. PET/CT may have a role in confirmation of suspected recurrence, but is not recommended for routine surveillance.

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In **chapter 5** we reviewed the role of FDG-PET/CT in staging and monitoring penile cancer. Combining functional with anatomical imaging, PET/CT outperforms both PET alone and CT alone in lymph node staging of many types of tumours. And although virtually all primary penile tumours are FDG-avid, PET/CT is not recommended for primary tumour staging as it has limited spatial resolution and urinary FDG excretion cause artefacts. The accuracy of PET/CT for lymph node staging seems to improve with the pre-test likelihood of metastatic nodes. In groins with normal physical examination, sensitivity is only 57%. In groins with palpably enlarged lymph nodes, sensitivity of PET/CT is up to 96%. For pelvic lymph nodes and distant metastases, PET/CT is more accurate if inguinal metastases are present. However, these results are based on a very limited number of studies. Overall, the role of PET/CT imaging in penile cancer remains ambiguous, especially in inguinal lymph nodes.

In **chapter 6** we conducted a retrospective study on the PET/CT scan. In our review we found suggestions that PET/CT may be particularly useful in detecting pelvic lymph node metastases and occult distant metastases prior to systemic chemotherapy and/or extensive surgery. In order to improve selection of patients that are most likely to benefit from such therapies, we looked at PET/CT results in patients with clinically overt lymph node metastases and/or large primary tumours (bulky T3 or T4).

FDG-PET/CT has a high sensitivity and negative predictive value (NPV) for staging of pelvic lymph nodes (sensitivity 85%, specificity 75%, NPV 90%, positive predictive value (PPV) 65%). FDG-PET/CT also has high PPV for detection of distant metastases (93%), which were found in 23% of patients. With these test properties, FDG-PET/CT enables early selection for multimodal treatment of patients with pelvic metastases, and may help avoid futile treatment of patients with distant metastases. These results are limited by the retrospective design and the lack of direct comparison to CT scanning alone.

Patients requiring extensive treatment are studied in **chapter 7 and 8**. The treatment of locoregionally advanced penile squamous cell carcinoma (LAPSCC) is challenging. Due to the rarity of the disease and especially advanced stages of the disease, the exact role of extensive surgery and chemoradiation therapy is not well established. Furthermore, major surgery inevitably leads to large defects requiring reconstructive surgery. Rectus abdominis myocutaneous (RAM) and abdominal advancement flaps have an independent and constant blood supply, are (relatively) easily harvested, and provide substantial skin coverage and soft

tissue. Chemoradiotherapy (CRT) is applied in squamous cell carcinoma from other organ sites, but its value has hardly been studied in penile cancer.

We retrospectively described the surgical and oncological outcomes in fifteen patients with LAPSCC undergoing surgical resection with RAM flaps or advancement flaps in **chapter 7**. Of fifteen patients, ten were treated with curative intent. Thirteen patients received induction chemotherapy. Thirteen patients (87%) experienced wound complications, including five Clavien-Dindo grade III complications. In eleven of the fifteen patients (73%), the disease recurred (median recurrence-free interval 106 days). The majority of recurrences (91%) were locoregional, and in four cases the patient also had lesions in distant organs. Ten of the 15 patients (67%) died of their disease. The overall median follow-up interval was 10.5 months. The results of this study show that large surgical procedures with reconstruction for LAPCC are associated with a risk of perioperative complications, including severe complications, and a cure rate of only 27%. In fact these results indicate that surgery needs careful consideration, and that there is a need for alternative treatments. Lack of robust quality-of-life-data is also a serious shortcoming in the decision process for this patient category. The study was limited by its retrospective design, the absence of quality-of-life measurements, and the cohort size.

In **chapter 8** preliminary results are provided of a prospective cohort study aiming to evaluate the efficacy and toxicity of CRT for LAPSCC. We included patients fit for CRT with LRAPC with no evidence of distant metastases. Locally advanced was defined as a cT4, tumours with satellite lesions, tumours with a very large surface, tumours in the penile basis/bulb or tumours where radical resection was deemed unattainable. Regionally advanced tumour was defined as palpable nodes >3cm in diameter (cN2-3), fixed nodes or suspicion of extranodal extension (ENE) on imaging, or pelvic nodal involvement (cN3). The CRT treatment scheme was adapted from the protocol for anal SCC. consisting of 49.5 Gy in 33 daily fractions on affected inguinal and pelvic areas, combined with mitomycin C (intravenous injection on day one) and capecitabine (orally, twice daily) on radiation days. Primary tumours and PET/CT-positive tumour deposit received a higher dose of 59.5 in daily fractions of 1.8 Gy. Patients with incomplete responses were eligible for salvage surgery or palliative therapy.

Forty patients were included. One-year and two-year progression free survival (PFS) were both 32%, overall survival (OS) was 70% and 52% respectively (median follow-up of 27 months). Upon first evaluation, responses were complete

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(35%), partial (35%) and progressive disease (30%). At the end of follow-up, sixteen patients (40%) are alive with no evidence of disease. In general, CRT was well-tolerated. Sixteen patients (40%) experienced one or more therapy related severe toxicity (all grade 3) during therapy or follow-up. No therapy related grade 4 or 5 events occurred. Disease recurred or progressed in 26 patients. This study is limited by its small sample size and lack of direct comparison to treatment alternatives. With these acceptable survival and toxicity rates, CRT appears as a feasible therapy for locoregionally advanced penile cancer. It reduces the necessity of surgical resection and can result in long-term disease-free survival.

Chapter 9 provides a reflection on the findings of the research presented in this thesis and places our results in a larger perspective.

SAMENVATTING

Peniskanker wordt in **hoofdstuk 1** geïntroduceerd met een korte omschrijving van de epidemiologie, etiologie en risicofactoren, tumorimmunologie, beeld- vorming en behandeling van de ziekte.

Hoofdstuk 2 en 3 behandelen immunologische aspecten van peniskanker. We vergeleken expressie van verscheidene immuunfactoren met ziekte-specifieke overleving (ZSO) en lymfekliermetastasen (LKM) bij diagnose. Daartoe gebruikten we gearchiveerd tumormateriaal. De rol van PD-L1 werd bestudeerd in **hoofdstuk 2**. PD-L1 is een zogenoemd "checkpoint" (controlepunt)-molecuul van het immuunsysteem dat tot expressie gebracht kan worden op tumorcellen of immuuncellen. Het remt de functie van T-cellen wanneer het zich bindt aan zijn receptor. Anti PD-L1/PD-1 immunotherapie kan dit mechanisme blokkeren en zo de aanval op de tumor door T-cellen versterken. We onderzochten PD-L1 expressie op verschillende plekken in de tumor-micro-omgeving (TMO): tumor, stroma en tumor-infiltrerende macrofagen (TIM). Ook noteerden we het patroon van PD-L1-expressie, zijnde diffuus of voornamelijk aan de tumor-stromarand.

Van de 200 tumoren waren er 96 (48%) PD-L1$^+$ (score 1% of hoger). Van de PD-L1$^+$ tumoren toonde er 59 (62%) voornamelijk randexpressie en 79 (82%) waren hoog-risico HPV (hrHPV) negatief, vergeleken met 75% van het totale cohort (p = 0,03). In vergelijking met PD-L1-negatieve tumoren, hadden de expressiepatronen verschillende prognostische waarde, zowel in de hele groep als in de hrHPV-negatieve subgroep. In de multivariabele analyse bleek een randexpressiepatroon geassocieerd met afwezigheid van lymfekliermetastasen (odds ratio (OR) 0,4) terwijl diffuse expressie juist een associatie had met slechtere overleving (hazard ratio (HR) 2,58). Deze resultaten waren meer uitgesproken in de hrHPV$^-$ subgroep (OR 0,25 en HR 3,92).

In **hoofdstuk 3** werden meer immuunfactoren bestudeerd. Analyse omvatte eerder beschreven hrHPV-status, immunohistochemische scores van klassiek en niet-klassiek humaan leukocyt antigeen (HLA) klasse I, PD-L1, en nieuwe gegevens van TIM en cytotoxische en regulatoire T-cellen. Klinische en pathologische karakteristieken werden meegenomen, evenals uitgebreide opvolggegevens.

In de gehele groep (n=216) werden diffuse PD-L1 tumorcelexpressie, CD163$^+$ macrofaag-infiltratie, upregulatie van niet-klassiek HLA klasse I, en weinig infiltratie van stromale CD8$^+$ T-cellen allen geassocieerd met LKM. In het

multivariabele model bleef alleen PD-L1 expressie over als significante predictor voor LKM (OR 2,8, p = 0,05). Negatieve hrHPV status en diffuse PD-L1 tumorexpressie werden significant geassocieerd met slechte overleving en bleven dat ook na correctie voor klinische parameters (HR 9,7, p < 0,01 en HR 2,8, p = 0,03). PD-L1 was de enige immuunfactor met verschillende expressie in HPV$^+$ en HPV$^-$ tumoren, met meer expressie in de laatstgenoemde groep (p = 0,03).

In het HPV- cohort (n = 158) werden LKM geassocieerd met diffuse tumor-PD-L1-expressie, veel CD163$^+$ macrofageninfiltratie, en weinig stromale CD8$^+$ T-celinfiltratie. De eerste twee parameters werden ook gelinkt aan ZSO. In het multivariabel regressiemodel bleef diffuse PD-L1 expressie significant ongunstig voor ZSO (HR 5,0, p < 0,01).

Deze resultaten benadrukken de complexiteit van de tumor micro-omgeving in peniskanker en wijzen richting meerdere mogelijke aangrijpingspunten voor immunotherapie. De hier beschreven immuunfactoren kunnen helpen bij risicostratificatie en moeten worden bestudeerd in klinisch onderzoek met immunotherapie om uiteindelijk naar geïndividualiseerde behandeling te leiden.

Hoofdstuk 4 geeft een overzicht van stadiëring en beeldvorming van peniskanker. Volgens de beschouwde literatuur is T-stadiëring gestoeld op lichamelijk onderzoek, maar kan MRI de sensitiviteit verhogen waar nodig. N-stadiëring is het meest complex voor kleine metastasen omdat die makkelijk te missen zijn. Voor de detectie van kleine metastasen heeft de dynamische schildwachtklierprocedure (SWKP) de voorkeur omdat het accurate pathologische stadiëring betreft zonder de bijwerkingen en complicaties van inguinale lymfeklierdissectie (ILKD). Voorafgaand aan de SWKP dient een echogeleide cytologische punctie gedaan te worden om onontdekte macrometastasen te voorkomen. De cytologische punctie is ook waardevol bij het aantonen van metastasen in verdachte liezen. Niettemin kunnen kleine metastasen gemist worden met elke niet- of minimaal invasieve stadiëringsmethode. Men moet blijven proberen de fout-negatievenratio daarvan verder te reduceren.

Zeer verdachte liezen verdienen een ILKD vanwege de grote therapeutische waarde. Voor M-stadiëring is conventionele anatomische beeldvorming aanbevolen. Van FDG-PET/CT is een hoge sensitiviteit beschreven. Diens waarde wordt verder geëvalueerd in **hoofdstuk 5 en 6** voor zowel N- als M-stadiëring.

Follow-up berust op lichamelijk onderzoek en echogeleide cytologische punctie. PET/CT heeft mogelijk een rol in lokaliseren van vermoedelijke recidieven maar is niet aanbevolen voor routinematige controle.

Hoofdstuk 6 is een uitgevoerde retrospectieve studie naar de PET/CT scan. In onze review vonden we aanwijzingen dat PET/CT vooral bruikbaar kan zijn bij de detectie van bekkenkliermetastasen en occulte afstandsmetastasen voorafgaand aan systemische chemotherapie en/of uitgebreide chirurgie. Om de patiënten die het meest waarschijnlijk profijt hebben van zulke therapieën beter te selecteren, onderzochten we PET/CT resultaten die waren gemaakt bij patiënten met klinisch evidente lymfekliermetastasering en/of grote primaire tumoren (grote T3 of T4). FDG-PET/CT had een hoge sensitiviteit en negatief voorspellende waarde (NVW) voor het stadiëren van bekkenklieren (sensitiviteit 85%, specificiteit 75%, NVW 90%, positief voorspellende waarde (PVW) 65%). FDG-PET/CT had ook een hoge PVW voor detectie van afstandsmetastasen (93%) die werden gevonden in 23% van de patiënten. Met deze testeigenschappen maakt PET/CT het mogelijk patiënten met bekkenkliermetastasen vroeg te selecteren voor multimodale behandeling en helpt PET/CT vergeefse behandelingen van patiënten met afstandsmetastasen te voorkomen. De bevindingen worden beperkt door de retrospectieve aard van de studie en het gebrek aan directe vergelijking met enkel een CT scan.

In **hoofdstuk 7 en 8** bestuderen we patiënten die een uitvoerige behandeling behoeven. De behandeling van een locoregionaal gevorderd plaveiselcelcarcinoom van de penis (LGPCCP) is uitdagend. Omdat de ziekt erg zeldzaam is, en al helemaal gevorderde stadia, is de precieze rol van chirurgie en chemoradiotherapie daarvoor nog deels onduidelijk. Daarnaast leiden grote operaties onvermijdelijk tot grote defecten die op hun beurt reconstructieve chirurgie vereisen. Bij myocutane rectus abdominis (MRA) en abdominale opschuifplastieken is er een onafhankelijke en constante bloedtoevoer, de lappen zijn (relatief) gemakkelijk te oogsten, en voorzien in substantiële huidbedekking en weke delen massa. Chemoradiotherapie (CRT) wordt toegepast in plaveiselcelcarcinomen (PCC) van verschillende organen, maar de waarde ervan voor peniskanker is nog nauwelijks beschreven.

In hoofdstuk 7 beschreven we retrospectief de chirurgische en oncologische uitkomsten van vijftien patiënten met LGPCCP die behandeld werden met MRA- of opschuifplastieken. Van de vijftien werden er tien met curatieve intentie behandeld. Dertien patiënten kregen inductie chemotherapie. Dertien patiënten

(87%) kregen te maken met wondcomplicaties, waaronder vijf Clavien-Dindo graad III complicaties. Elf van de vijftien patiënten (73%) kreeg een recidief (mediane ziektevrij interval 106 dagen). De meeste recidieven (91%) was locoregionaal, in vier gevallen had de patiënt ook metastasen op afstand. Tien van de vijftien patiënten (67%) stierven als gevolg van de ziekte. Het totale mediane follow-up-interval was 10,5 maand. De resultaten van deze studie laten zien dat grote chirurgische ingrepen met reconstructie voor LGPCCP geassocieerd zijn met een risico op complicaties, waaronder ook ernstige complicaties, alsmede een genezingspercentage van slechts 27%. In feite wijzen deze resultaten erop dat chirurgisch ingrijpen zorgvuldig moet worden afgewogen en dat er behoefte is aan alternatieve behandelopties. Een gebrek aan robuuste kwaliteit-van-leven-data is ook een ernstig tekortkomen bij besluitvorming met deze patiëntengroep. Deze studie werd beperkt door haar retrospectieve aard en het ontbreken van kwaliteit-van-leven-metingen en de cohortgrootte.

In **hoofdstuk 8** geven we voorlopige resultaten van een prospectieve cohortstudie met als doel de effectiviteit en toxiciteit van chemoradiatie voor locoregionaal gevorderd peniscarcinoom te evalueren. We includeerden patiënten die fit genoeg waren voor CRT en definieerden LGPCCP zonder aanwijzingen voor afstandsmetastasen. Lokaal gevorderd werd gedefinieerd als cT4, tumoren met satellietlaesies, tumoren met een groot oppervlak, tumoren in de penisbasis of -bulbus, of tumoren waar radicale resectie anderszins onhaalbaar werd geacht. Regionaal gevorderd werd gedefinieerd als palpabele klieren met diameter >3cm (cN2/cN3), gefixeerde lymfeklieren of verdenking op extranodale groei (ENE) op beeldvorming, of bekkenkliermetastasering (N3).

Het CRT behandelschema was gebaseerd op het protocol voor anuscarcinomen, bestaande uit 49,5 Gy in 33 dagelijkse fracties op aangedane inguinale en iliacale gebieden, gecombineerd met mitomycine C (intraveneuze injectie op dag 1) en capecitabine (oraal, tweemaal daags) op bestralingsdagen. Primaire tumoren en PET/CT-positieve tumordeposities kregen een hogere dosis van 59,5 in dagelijkse fracties van 1,8 Gy. Patiënten met een incomplete respons kwamen in aanmerking voor aanvullende (salvage) chirurgie of palliatieve therapie.

Veertig patiënten werden geïncludeerd. 1-jaar en 2-jaar progressievrije overleving (PVO) waren beiden 32%, totale overleving (TO) waren respectievelijk 70% en 50% (mediane follow-up 27 maanden). Bij de eerste evaluatie werden responses gemeten als compleet (35%), partieel (35%) en progressieve ziekte (30%). Aan het einde van de follow-up waren zestien patiënten (40%) in leven

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zonder tekenen van ziekte. Over het algemeen werd CRT goed verdragen. Zestien patiënten (40%) kregen één of meer klachten van ernstige toxiciteit (allen graad 3) tijdens de behandeling of daarna. Er werden geen behandeling-gerelateerde graad 4 of 5 klachten gemeld. Die ziekte recidiveerde bij 26 patiënten (65%). Deze studie werd beperkt door de kleine studiegroep en de afwezigheid van een tweede arm met alternatieve behandeling. Met deze acceptabele overlevings- en toxiciteitscijfers lijkt CRT een geschikte behandeling voor locoregionaal gevorderd peniscarcinoom. Het vermindert de noodzaak tot chirurgisch ingrijpen en kan resulteren in een langdurige ziektevrije overleving.

In **hoofdstuk 9** reflecteren we op de bevindingen van het in dit proefschrift beschreven onderzoek. Daarnaast worden de resultaten in een bredere context geplaatst.

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Chapter 11

Appendices

LIST OF ABBREVIATIONS

AE	adverse event
ASA	American Society of Anesthesiologists
AUC	area under the curve
AWD	alive with disease
β2M	Beta-2-microglobulin
BMI	body mass index
CD	cluster of differentiation
CI	confidence interval
CRT	chemoradiotherapy
CTCAE	Common Terminology Criteria for Adverse Events
CT	computed tomography
DD / DOD	died of disease
DSS	disease specific survival
DSM	disease specific mortality
DSNB	dynamic sentinel node biopsy
DVT	deep venous thrombosis.
EAU	European Association of Urology
EGFR	epithelial growth factor receptor
ENE	extranodal extension
-f	free flap
FDA	food and drug administration
FDG	^{18}F-fluorodeoxyglucose
FFPE	formalin fixed and paraffin embedded
FN	false negative
FNAC	fine needle aspiration cytology
FNR	false negative rate
FP	false positive
FU	follow-up
Gy	gray
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HR	hazard ratio
hrHPV	high risk human papilloma virus (also oncogenic HPV)
ICG	indocyanine green
iChT	induction chemotherapy
IFN-γ	interferon gamma

IHC	immunohistochemical
ILND	inguinal lymph node dissection
IMRT	intensity modulated radiotherapy
IQR	inter quartile range
LAG3	lymphocyte activation gene 3 protein
LAPSCC	locoregionally advanced penile squamous cell carcinoma
LNM	lymph node metastasis or metastases
LOS	length of hospital stay
LS	lichen sclerosus
LVI	lymphovascular invasion
MCC	Moffit Cancer Center
MIL	modified inguinal lymfadenectomy
MRI	magnetic resonance imaging
N/A or NA	not applicable
NED	no evidence of disease
NCCN	National Comprehensive Cancer Network
NCI	Netherlands Cancer Institute (NL: Nederlands Kanker Instituut – Antoni van Leeuwenhoekziekenhuis)
NPV	negative predictive value
NOS	not otherwise specified
NSCLC	non small cell lung cancer
OR	odds ratio
ORAM	oblique rectus abdominis myocutaneous flap
OS	overall survival
PCR	polymerase chain reaction
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PeIN	penile intraepithelial neoplasia
PET	positron emission tomography
PERCIST	positron emission tomography (PET) response criteria in solid tumors
PFS	progression free survival
PLND	pelvic lymph node dissection
PPV	positive predictive value
PROM	patient reported outcome measure
PRT	postoperative radiotherapy
Pt	patient
QOL	quality of life
RAM	rectus abdominis myocutaneous flap
RFS	recurrence free survival

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ROC   receiver operating characteristic

RS   recurrence site

SCC   squamous cell carcinoma

SIB   simultaneous integrated boost

SNB   sentinel node biopsy

SPECT   single photon emission computed tomography

TFL   tensor fasciae latae muscle flap

TFM   tumour-free margins

TIM   tumor infiltrating macrophage

TNM   Tumor Node Metastasis

TME   tumor microenvironment

TN   true negative

TP   true positive

TPF   docetaxel, cisplatin, fluorouracil

Treg   regulatory T-cell

UICC   Union for International Cancer Control

US   Ultrasound

USA   United States of America

UTI   urine tract infection

-v   vascularized

VAS   visual analogous scale

VRAM   vertical rectus abdominis myocutaneous flap

WHO   world health organization

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PUBLICATIONS & PRESENTATIONS

Journal publications

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2. **Ottenhof SR**, Djajadiningrat RS, Versleijen MWJ, Donswijk ML, van der Noort V, Brouwer OR, Graafland NM, Vegt E, Horenblas S. F-18 Fluorodeoxyglucose Positron Emission Tomography with Computed Tomography Has High Diagnostic Value for Pelvic and Distant Staging in Patients with High-risk Penile Carcinoma. Eur Urol Focus. 2021 Mar 5:S2405-4569(21)00055-9. doi: 10.1016/j.euf.2021.02.012. Online ahead of print.

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- Epidemiology of Penile Cancer. Sarah R. Ottenhof, Maaike C. G. Bleeker, Daniëlle A. M. Heideman, Peter J. F. Snijders, Chris J. L. M. Meijer, Simon Horenblas. Pages 1-9.
- Etiology of Penile Cancer. Sarah R. Ottenhof, Maaike C. G. Bleeker, Daniëlle A. M. Heideman, Peter J. F. Snijders, Chris J. L. M. Meijer, Simon Horenblas. Pages 11-15.
- Management of Lymph Nodes. Sarah R. Ottenhof, Niels M. Graafland, Simon Horenblas. Pages 167-1994.
- Follow-Up of Patients with Penile Cancer. Simon Horenblas, Sarah R. Ottenhof, Niels M. Graafland. Pages 303-309.

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- Dynamic Sentinel Node Biopsy and FDG-PET/CT for Lymph Node Staging in Penile Cancer. Niels M. Graafland, Sarah R. Ottenhof, Renato A. Valdés Olmos, Erik Vegt. Pages 45-53

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- Penis Cancer. S.R. Ottenhof and A. Parnham, 2018. Access via https://patients.uroweb.org/library/penis-cancer/
- Penile Curvature / Peyronie's disease. M. Behrent, S.R. Ottenhof and G. Patruno. Access via https://patients.uroweb.org/library/penile-curvature/

Presentations

1. Externe Refereeravond Urologie, Breda. Peniscarcinoom, nieuwste inzichten. 10 nov 2020
2. Bijeenkomst Taskforce Nederland HPV kankervrij, Rotterdam. Urologie en HPV. 14 feb 2020
3. EAU Annual Congress (EAU18), Kopenhagen, Denemarken. Tumour immunology of penile cancer (poster) 16 mrt 2018
4. EAU Annual Congress (EAU18), Kopenhagen, Denemarken. Use of FDG-PET/CT for staging pelvic lymph nodes (poster) 16 mrt 2018 ☆ Best poster of the session

5. NVU najaarsvergadering, Nieuwegein. Het gebruik van PET/CT-scans voor het stadiëren van bekkenklieren van peniscarcinoompatiënten met lieskliermetastasen. 3 nov 2017

6. Dutch Tumor Immunology Meeting, Breukelen. Tumor escape in the microenvironment of penile carcinoma. 22 juni 2017

7. American Urological Association Annual Meeting (AUA2017), Boston, USA. Tumor escape in the microenvironment of penile carcinoma (poster). 13 mei 2017

8. Refereeravond UMCU, Utrecht. Beeldvorming en stadiëring van peniscarcinomen. 18 apr 2017

9. EAU Section of Urological Research (ESUR16), Parma, Italy. Tumour escape in the microenvironment of penile carcinoma. 22 okt 2016

10. Onderzoeksschool Oncologie Amsterdam retreat 2016, Renesse. Tumour escape in the microenvironment of penile carcinoma. 5 okt 2016
☆ 2e prijs voor beste presentatie

11. NVU voorjaarsvergadering, Leiden. Architectuur van het corpus spongiosum. 20 mei 2016
☆ Genomineerd voor de Vlietstraprijs

12. NVU voorjaarsvergadering, Leiden. Immunologie van het peniscarcinoom. 20 mei 2016

13. EAU Annual Congress (EAU16), München, Duitsland. Immunotherapy in Penile Carcinoma - Programmed Death Ligand 1 is a potential target (poster). 12 mrt 2016
☆ Best poster of the session

14. 28e Symposium voor Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Leiden. De architectuur van het corpus spongiosum. 11 dec 2015

15. NVU najaarsvergadering, Nieuwegein. Programmed Death Ligand 1 is een mogelijk aangrijpingspunt voor immunotherapie in peniscarcinoom. 6 nov 2015

16. OOA retreat 2015, Renesse. Immunotherapy in Penile Carcinoma – PD-L1 is a potential target (poster) 14 okt 2015

17. Refereeravond UMCU, Utrecht. De structuur van het corpus spongiosum urethrae, eerste resultaten van een anatomische studie. 10 feb 2015

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ABOUT THE AUTHOR

Sarah Rosanne Ottenhof was born to Britta Verboom and Ferdinand Ottenhof on the 8th of July in Wageningen, the Netherlands. She grew up with her elder sister Eva and two elder brothers Anne and Maarten.

After graduating from secondary school (Karel de Grote College, Nijmegen) she travelled to Central America where she learned Spanish and participated in a cultural association in Honduras. In 2007 she started medical school at University Utrecht.

Her interest in urology was born during a surgical rotation and she continued with a combined clinical and research internship at the urology department of the University Medical Center Utrecht under the supervision of drs. MTWT Lock, prof. dr. LMO de Kort and prof. dr. RLAW Bleijs. The results of that anatomical study were published in the Journal of Urology. During her medical training Sarah also went abroad to Argentina (ENT medicine) and Ghana (anaesthesia/urology).

After receiving her medical degree she started her PHD training at the Netherlands Cancer Institute in 2015 under the supervision of prof. dr. S Horenblas and dr. ES Jordanova. Her research mainly focused on immunological aspects of penile cancer, staging and imaging of penile cancer, and the evaluation of old and new therapies for advanced stages of the disease. In addition to scientific journal papers Sarah contributed to books on penile cancer and wrote patient information leaflets as a member of the working group on patient information of the European Association of Urology (EAU).

In July 2018 Sarah started working as a medical officer (resident not in training, ANIOS). She worked at the urology departments of Tergooi hospital, Sophia Children's hospital and Amphia hospital. She is currently appointed as surgical resident in training at the IJsselland hospital in Capelle aan den IJssel as part of her urology training at the Erasmus Medical Center in Rotterdam.

She is happily together with Jannes ten Berge, they currently live in Rotterdam.

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