Diplomarbeit

Penile Squamous Cell Carcinoma: A Disease with a Bimodal Pathway and a Clinicopathological Prognostic Challenge

eingereicht von

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unter der Anleitung von

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Ort, Datum …….. (Unterschrift)

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Preface

In summer 2009 I passed a medical clerkship of 6 weeks duration at the Institute of Pathology in Villach, Carinthia. My supervisor, DR. Ute Fillipot, who always had a sympathetic ear for me, gave me a helping hand to come in contact with the Institute of Pathology in Graz. There, I came to know Dr. Sebastian Mannweiler and Dr. Sigrid Regauer. They offered me to take part in a study of penile carcinogenesis and -in this context- the opportunity to begin my diploma project. The aim was to elucidate the major pathways of penile carcinogenesis in Austria. In archival specimens of the last 25 years, we examined the distribution of HPV genotypes in HPV-induced penile squamous cell carcinomas and in HPV-negative penile cancers. We placed special emphasis on chronic inflammatory skin diseases such as lichen sclerosus and lichen planus. During reclassification of the archival surgical specimens according to the up-dated TNM classification (2009) we realized that the subclassification of pT1-carcinomas into pT1a and pT1b carcinomas did not correlate with the presence of lymph node metastases. So, the idea for the second study was born: to evaluate the new TNM classification for its predictive value for correctly predicting lymph node metastases.

During the 2 years I worked on the diploma project at the Institute of Pathology in Graz, two original publications have been accepted for publication.

The first manuscript, where I am co-author, has been published in The American Journal Surgery Pathology.
The second manuscript has been published in Urologic Oncology.
In the second manuscript, the first and second author, Dr. Sebastian Mannweiler and Stephan Sygulla, cand. med., have contributed equally to this work.

The diploma thesis consists of both published manuscripts with an expanded introduction.

Acknowledgements

Special thanks goes to my supervisor Prof. Dr. Sigrid Regauer who supported me right from the beginning.

Thanks to the head of the Institute of pathology in Graz, Sytria, who financed my trip to Leipzig, Germany, where I presented the results of the second part of my diploma project on an international conference.

Thanks to Dr. Sebastian Mannweiler and Dr. Yas Razmara for their support and cooperation.

Thanks to my family and all my friends, in particular to my girlfriend Monika, for their support and supportive words.

Zusammenfassung

Peniskarzinome kommen in Afrika und Südamerika häufig, in USA und Europa mit geringer Inzidenz unter 1% vor. Erstmalig in Österreich werden Ergebnisse zur penilen Karzinogenese aus der Steiermark, einer Region mit niedriger Inzidenz, präsentiert. Transformierende Infektionen mit high-risk Genotypen und die Dermatosen *Lichen sklerosus* und *Lichen planus* sind die zwei wichtigsten Ursachen, wobei die exakte Karzinogenese aber noch unklar ist. Peniskarzinome werden nach der TNM Klassifikation klassifiziert, doch ist die korrekte Vorhersage von Lymphknotenmetastasen schwierig und die Morbidität unnötiger inguinaler Lymphadenktomien groß. In dieser Arbeit untersuchten wir die Häufigkeit und Verteilung von HPV-HR Genotypen mit transformierender Infektion und von Dermatosen in Peniskarzinomen der Steiermark Österreich, wie auch die prognostische Wertigkeit der revidierten TNM-Klassifikation (2009) bzgl. der korrekten Vorhersage von Lymphknotenmetastasen. Insgesamt wurden 164 Formalin-fixierte Präparate aus dem Archiv des Instituts für Pathologie der Medizinischen Universität Graz untersucht. Etwa 60% waren durch HPV verursacht. Davon waren 91% transformierende Infektionen mit nur einem Genotyp (HPV16:80%, HPV33:6%, HPV18:3%, HPV45:2%). Multiple Genotypen wurden in 9% nachgewiesen. 40% aller Karzinome waren HPV-negativ, waren in den fortgeschrittenen pT2 / pT3 Tumorstadium überrepräsentiert. In 2/3 wurde Lichen sklerosus und einem Drittel Lichen planus nachgewiesen. Auffällig war ein dichtes lymphozytäres Infiltrat mit Ansammlung von Lymphozyten mit einem monoklonal rearrangierten Gamma Locus. Im zweiten Teil der Studie wurden 4 pT1 Klarzellkarzinome und 72 pT1 Plattenepithelkarzinomen auf prognostische histologische Kriterien untersucht. Klarzelldifferenzierung war statistisch signifikant mit Lymphknotenmetastasen (100% Klarzell- vs. 11% Plattenepithelkarzinomen) und schlechterem Überleben (50% Klarzell- vs. 5.5% Plattenepithelkarzinomen) assoziiert. Der einzige statistisch signifikante prädiktive Faktor für Lymphknotenmetastasen in den Plattenepithelkarzinomen war der Nachweis von Lymphgefäßeinbrüchen im Primärtumor mit 100% Spezifität. Weder die revidierte TNM-Klassifikation, noch der Tumorgrad, perineurale Invasion, Invasionstiefe, Wachstumstyp oder HPV-Status konnten Metastasen korrekt vorhersagen. Diese beiden Arbeiten zeigen, daß 1.) etwa 50% der steirischen Peniskarzinome durch präventive Impfung gegen HPV16/18 vermeidbar gewesen wären, 2.) In HPV- negativen Dermatosen-assoziierten Karzinome die Immundysregulierung eine Rolle in der Karzinogenese spielen könnte, und 3) Männer mit Klarzellkarzinomen und Lymphgefäßinvasion von einer elektiven Lymphknotendissektion profitieren würden.

Abstract

Penile cancers are rare malignancies with an incidence lower than 1% in Europe and USA, compared to high incidence countries such as Africa and South America with incidences up to 10%. This is the first study in the state of Styria, Austria, a low-incidence area for penile cancer investigating the two major pathways of penile carcinogenesis, e.g. transforming Human papilloma virus (HPV) infections and penile cancers arising in lichen sclerosus and lichen planus. The exact role of dermatoses is unclear. The most widely used classification system for penile cancer is the TNM classification, but reliable detection of lymph node metastases in early stage carcinomas is difficult. First, we analyzed the distribution of transforming HPV infections and dermatoses in penile cancers. Secondly, we tested the revised TNM classification (2009) for its predictive accuracy with respect to lymph node metastases. We examined 164 archival formalin-fixed specimens from the Institute of Pathology, Medical University Graz, Styria. About 60% of penile cancers were induced by HPV-high-risk genotypes, with 91% being induced by a single genotype (HPV 16:80%, HPV 33:6%, HPV 18:3%, HPV 45:2%) and 9% by multiple genotypes. Lichen sclerosus could be observed in two thirds of HPV-negative penile cancers and lichen planus in the remaining one third. HPV-negative cancers were overrepresented in large (pT2 / pT3) tumor stages. About half of the HPV-negative lesions showed a monoclonally rearranged T-cell receptor gamma locus in the T-cell dominant lymphocytic infiltrates. Of the 76 pT1, 72 carcinomas were squamous cell carcinomas and 4 clear cell carcinomas. 100% of patients with clear-cell carcinomas, but only 11 % with squamous cell carcinomas had metastases. Patients with clear-cell carcinomas had a poorer survival than patients with squamous cell carcinomas (50% versus 5%). In squamous cell cancers, lymphatic invasion was the only independent predictive factor for lymph node metastases. Neither the revised TNM-classification, nor tumor grade, perineural invasion, depth of invasion, growth pattern and HPV-status could predict metastases. In summary, presently available vaccinations against HPV high-risk 16/18 could have prevented approximately 50% of Styrian penile cancers. Immune dysregulation most likely contributes to the development of HPV-negative penile cancers, and early recognition and treatment of dermatoses may prevent these cancers. Clear cell carcinomas are more aggressive than squamous cell cancers. Patients with clear-cell carcinomas and patients with lymphatic invasion in squamous cell cancers may benefit from an elective lymphadenectomy.

Index

Glossar and Shortcuts

CIN ..... Cervical Intraepithelial Neoplasia
d-PeIN..... differentiated Penile Intraepithelial Neoplasia
HE..... Hematoxylin and Eosin
HPV ..... Human Papilloma Virus
LP ..... Lichen Planus
LS .....Lichen Sclerosus
PeIN.....Penile Intraepithelial Neoplasia
Rb .....Retinoblastoma Gene
SCC..... Squamous Cell Carcinoma
VIN .....Vulvar Intraepithelial Neoplasia

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1 Introduction

Invasive penile cancer accounts for 0.4% to 0.6% of all male malignancies in the United States and Europe, [1,2] which are low-incidence areas for penile cancer, compared with 10% in high-incidence countries of Africa and South America [3,4]. Investigations on penile carcinogenesis in high-incidence areas are numerous. In Austria, penile cancers are rare diseases with an incidence lower than 1%. There are no publications about penile carcinogenesis from Austria. Worldwide, transforming human papilloma virus (HPV) high-risk infections are responsible for about half of all penile cancers. HPV-negative penile cancers often arise in the background of dermatoses, in particular lichen scloerosus (LS) and lichen planus (LP).

1.1 Aim of this Project

This is the first study in Austria, a low incidence country for penile cancers, investigating the bimodal pathway of penile carcinogenesis, e.g. HPV-infection and dermatoses in penile cancers. In this diploma project, we analyzed penile precursor lesions and invasive penile cancers diagnosed in the state of Styria during the past 25 years. The project was divided into two parts. The first aim was to examine the distribution of HPV genotypes in penile cancers in Styria compared to the general distribution worldwide. Special emphasis was placed on the documentation of LS and LP when present. The second aim was to correlate the prognostic value of the most recent revision of the widely used TNM classification system (2009). In particular, we were interested if in patients with early stage pT1 penile carcinoma the presence of lymph node metastases could be predicted correctly based on histological factors.

1.2 Classification of Penile Diseases

Penile diseases can be categorized in benign, premalignant and malignant lesions. The benign diseases can be divided into chronic inflammatory diseases such as LS and LP, benign tumors such as fibromas, hemangiomas, melanocytic nevi and leiomyomas and viral infections [5]. Depending on the etiology, the precursor lesions are divided into HPV-induced intraepithelial neoplasia (PeIN) and HPV-negative differentiated PeIN (d-PeIN). The great majority of malignant tumors are penile squamous cell carcinomas (SCC), followed by

adenosquamous carcinomas, basal cell carcinoma, Paget disease, malignant melanoma, angiosarcoma, leiomyoma, epitheloid sarcoma and lymphoma [5].

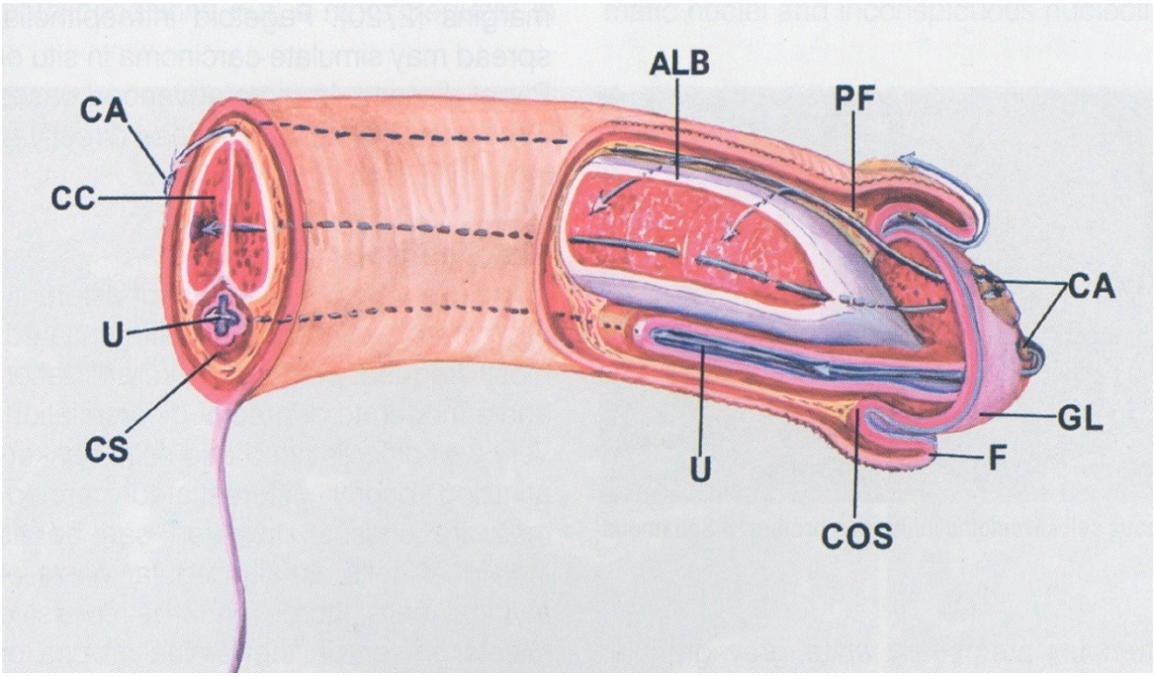
1.3 Penile Anatomy

Relevant aspects of the penile anatomy are important for diagnosis, classification and treatment of penile malignancies [3]. The following pages should give a brief overview. The penis is composed of the penile body with erectile tissues, urethra, glans penis and foreskin [5].

Penile Body:

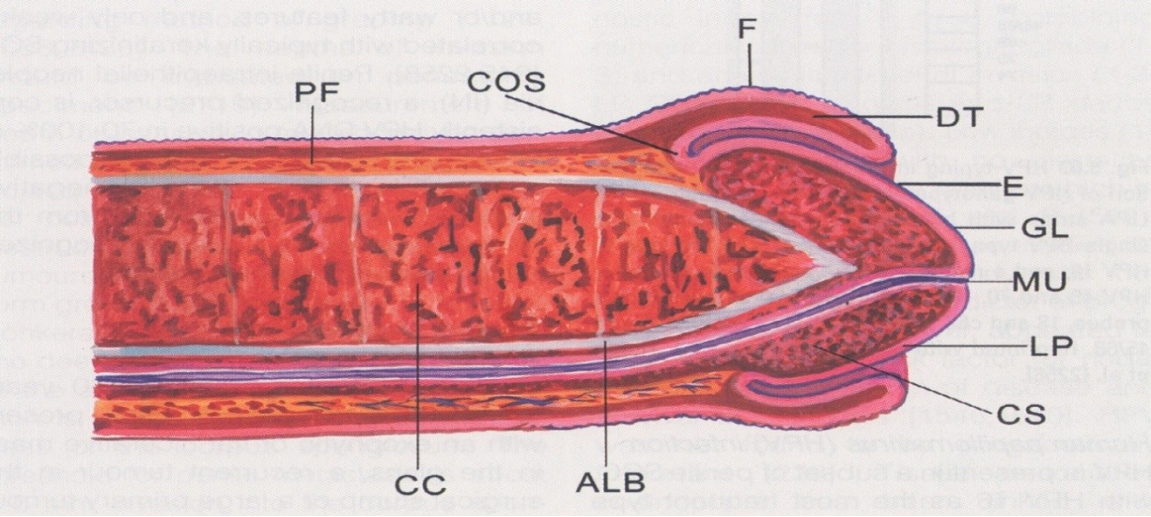
The penile body (Fig.2) harbours three cylinders of erectile tissue, which are two corpora cavernosa and the corpus spongiousum. The corpora cavernosa are separated by a median raphe. The latter is continous with the Tunica albuginea, which covers both corpora cavernosa and corpus spongiosum. In turn, the Tunica albuginea is encased in the penile Buck fascia which is highly vascular and built of loose connective tissue. Through the penile fascia, Tunica albuginea and the erectile tissues small nutrition vessels and adipose tissue are crossing through which cancers spread from the penile fascia to the erectile tissues (Fig.1). This is also a frequent site and positive resection margin[5].

Figure 1: Routes of Local Spread of Penile Cancer



Lines and arrows represent pathways of local tumor (CA) spread from: distal glans (GL), foreskin (F),coronal sulcus (COS) to proximal corpus spongiosum (CS),corpora cavernosa (CC), penile fascia (PF), skin & urethra (U).Tunica albuginea (ALB) Source: WHO Tumours of the Urinary System and Male Genital Organs [4]

Figure 2: Penile Structures



Penile structures in alphabetical order: Tunica albuginea (ALB), corpus cavernosum (CC), coronal sulcus (COS), corpus spongiosum (CS), Dartos fascia (DT), epithelium (E), foreskin (F), glans (GL), lamina propria (LP), meatus urethrae (M), and penile fascia (PF) Source: WHO Tumours of the Urinary System and Male Genital Organs [4]

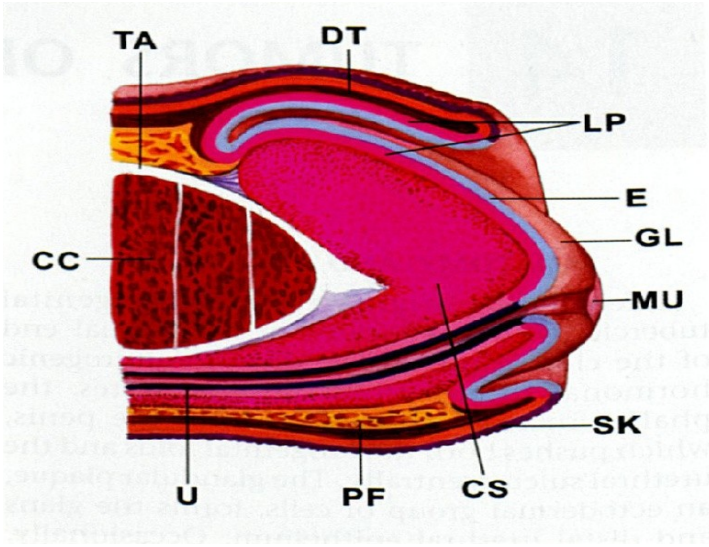
The penile urethra is centrally located in the corpus spongiosum. The distal 5 to 6 mm end of the urethra with the fossa navicularis shows a non-keratinizing glycogenated squamous epithelium. In and around the urethra periurethral mucinous (Littrè) glands and prostate-specific antigen (PSA)-positive prostatic-like glands and ducts have been described. As epithelial alterations can be found in the penile urethra in the background of penile cancer, it may happen that primary urethral tumors are confused with penile neoplasms. If a squamous epithelium arises in regions other than the distal portion of the urethra it should be considered as metaplastic. Furthermore, the urethra is a common site of a positive resection margin in partial penectomies [5].

Glans Penis and Foreskin

The anterior penis (Fig.3/4) consists of the glans penis with corpus spongiosum as its main component, the coronal (balanopreputial) sulcus and the foreskin. At the base of the glans, the frenulum is attached to the foreskin. The following anatomic layers are identified: a mucous membrane, which consists of squmaous epithelium and lamina propria, corpus spongiosum, Tunica albuginea, and corpora cavernosa [5]. The uppermost layer of the glans is a nonkeratinizing and glycogen enriched squamous epithelium in uncircumcised and keratinizing squamous mucosa in circumcised men. Most neoplasms of the penis originate in this epithelium. The lamina propria consists of loose connective tissue and separates the epithelium from the corpus spongiosum. It harbors lymphatic vessels, peripheral nerves, and occasional Vater Paccini corpuscles [5]. In two thirds of penectomy specimens, the Tu-

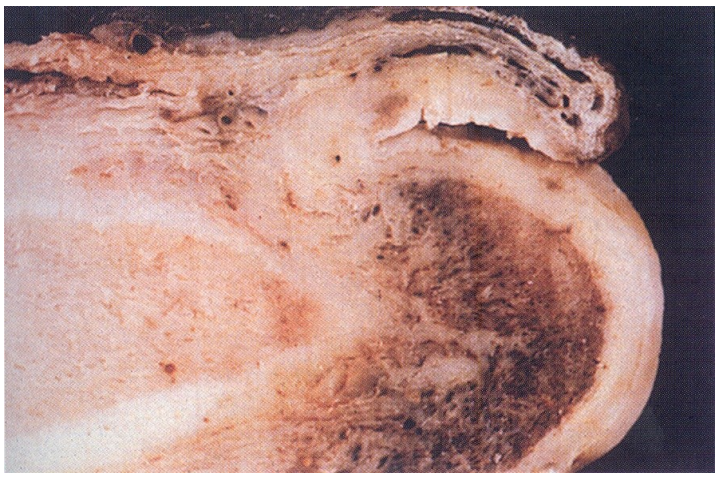
nica albuginea and corpora cavernosa are part of the glans tissues. In the remaining third, these structures are restricted to the shaft. The Tunica albuginea completely encases the corpus spongiosum and acts as a natural barrier against the spread of cancer. In the distal portion of the penis most epithelial tumors occur. The glans penis and the foreskin join at the coronal sulcus. This is also a typical location for primary penile carcinomas and in cases of primary foreskin carcinomas a positive margin [5].

Figure 3: Schematic Cross Section through Glans Penis



Shortcuts in alphabetical order: CC:corpus cavernosum, CS:corpus spongiosum, DT: Tunica dartos, E:epithelia, GL:glans penis, LP:lamina propria, MU:meatus urethrae, PF:Buck penile fascia, SK:skin, TA: Tunica albuginea, U:urethra
Source: AFIP Atlas [5]

Figure 4: Cross Section through Formalin-fixed Glans Penis Specimen



Source: AFIP Atlas [5]

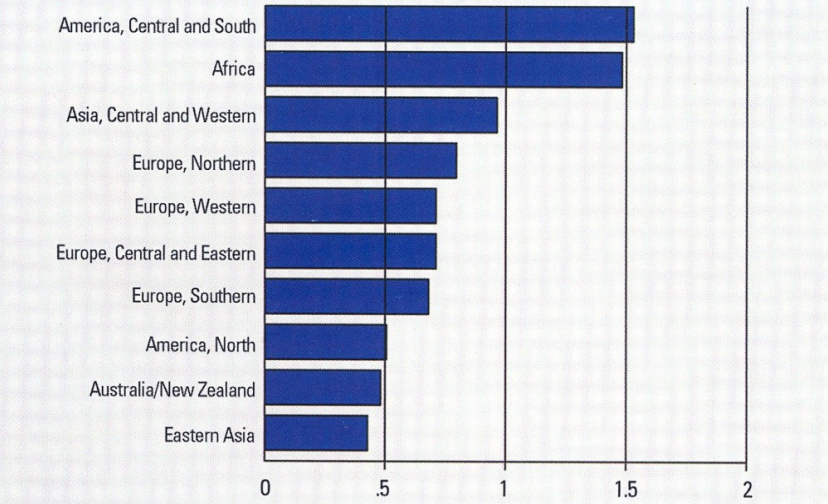
The foreskin covers the glans with an inner and outer lamina. The outer skin is dark and wrinkled and the inner mucosal surface is pale and smooth and consists of keratinizing mucosa with sebaceous glands. Most carcinomas of the foreskin arise in the inner mucosal

surface. Foreskins differ in length. This diversity is considered to play an important role in the pathogenesis of the penile cancer, as an association between long and phimotic foreskins and penile cancer is recorded [5].

1.4 Penile Cancer

Invasive penile cancer is a rare malignancy which occurs in different regions in different incidences (Fig.5). Invasive penile cancer accounts for 0.4-0.6% of all male malignancies in the USA and Europe [1,2], which are low incidence areas for penile cancer, compared to 10% in high incidence countries of Africa and South America (Fig. 5) [6-8].

Figure 5: Different Incidences Worldwide

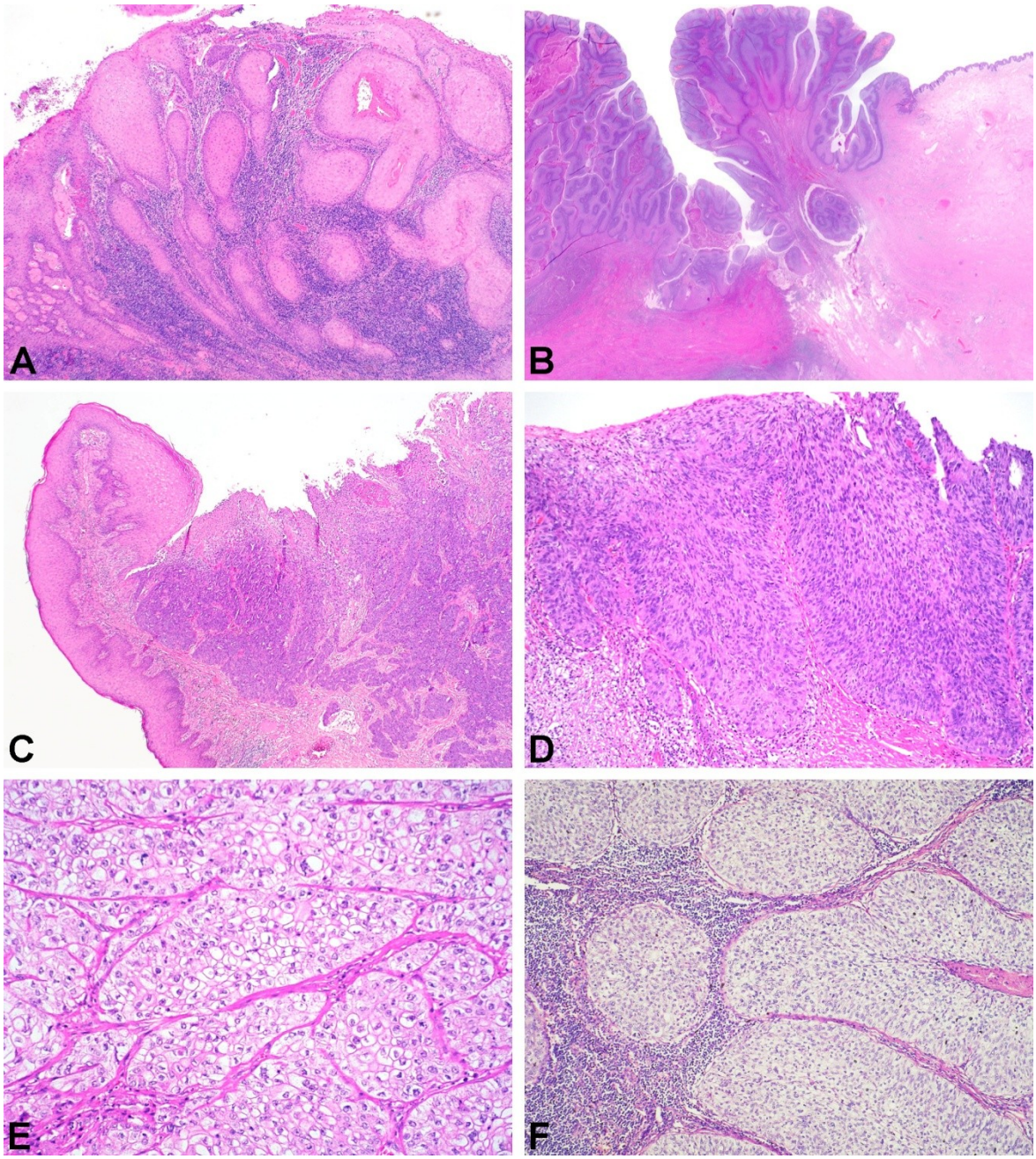


Source: WHO Tumours of the Urinary System and Male Genital Organs [4]

1.4.1 Histologic Subtypes of Penile Carcinomas

The most frequent subtype of penile cancer is the SCC with the following histological variants: basaloid, verrucous, warty, papillary, sarcomatoid, pseudoglandular, pseudohyperplastic SCC and adenosquamous SCC [5]. In addition, clear cell carcinomas, which are exclusively rare, are illustrated below because they are relevant for this diploma project according to the prediction of lymph node metastases. They are exophytic, solid, highly vascularized, mitotically active tumors arising at the inner side of the foreskin and consist of > 80% of clear cells. They are believed to arise from clear cells of adnexal structure of foreskin and submucosal glands. Lymph node metastases of clear cell carcinomas are composed also predominantly of clear cells (for details see [9]).

Figure 6: Histologic Variants of Penile Cancer



A: well differentiated keratinizing SCC; B: condylomatous SCC, C and D: basaloid SCC Source: Dr. Regauer, Institute of Pathology, Medical University Graz

E: Clear cell carcinoma; F: lymph node metastasis of clear cell carcinoma. Source: Liegl and Regauer from American Journal Surgical Pathology [9]

1.4.2 Risk Factors for Penile Cancer

In the older literature, infections such as Chlamydia trachomatis and Neisseria gonorrhea and photodynamic therapy have been mentioned as risk factors for developing penile cancer [4,10]. Other risk factors included warts/HPV related lesions, LS, smoking, injury tears, balanitis, and immunosuppression [5]. Warm climate, tropical regions, poor hygiene and poverty were thought to be factors in high incidence areas. Genetic factors appear less im-

portant than environmental factors but some authors discussed familial occurrence. A Swedish study revealed a significantly increased risk for developing penile squamous cell carcinoma if a sibling or parent was affected with penile cancer [5].In regions with a low socioeconomic status a higher incidence was reported. A study concentrated on examining the risk dependent on the poverty level and reported a 43 percent higher risk for penile cancer in patients from countries with 20 percent or more of the population living at or below poverty level as compared to patients living in regions with less than 10 percent poverty [5]. Phimosis is a clinical condition, in which the foreskin cannot be retracted. It is often associated with chronic balanitis or LS and HPV-negative penile cancer. A Brazilian study revealed that 60 percent extent of males with phimosis showed penile cancer. Interestingly, most authors agree that the circumcision protects against invasive cancer. Cigarette smoking or chewing tobacco has been also associated with penile cancer. The risk of penile squamous cell carcinoma increases in smokers versus nonsmokers with pack-years. Tobacco-specific nitrosamine that is present in smokers' urine, salvia and inner surface "preputial secretion", is believed to be responsible for the increased risk [5]. Overall, there is good evidence for only two major pathways of penile carcinogenesis [4,10]. On the one hand, transforming infections with HPV high-risk genotypes are responsible for a significant number of penile cancers, although not to the extent observed in cervical squamous cell carcinomas, and on the other hand, HPV-negative penile carcinomas associated with dermatoses such as LS and LP [8,11].

1.5 HPV Infections

HPV belongs to the papilloma virus family [12]. They infect basal keratinocytes of the skin or mucous membranes in the anogenital region and oropharynx [13]. HPV is the most common sexually transmitted disease worldwide [14] and plays an essential role in various cancers in both sexes [12]. It is estimated, that up to 75% of adults will have contact with HPV during lifetime [15]. Not every infection causes cancer since the immune system is capable of clearing the infection. According to the risk of developing cancer, HPV genotypes can be divided into non-oncogenic HPV genotypes producing verruca vulgaris and oncogenic HPV genotypes.

1.5.1 Oncogenic HPV Genotypes

Oncogenic genotypes can be subdivided into high-risk and low-risk genotypes [12]. The HPV low-risk genotypes are seldom linked to cancer. The main representative genotypes are HPV 6/11 causing lesions in the female or male anogenital region which are referred to as

condyloma accuminatum or genital warts. The group of HPV high-risk genotypes with main representative genotype HPV 16/18 is characterized by a strong link to cancer. These HPV high-risk genotypes are involved in cancer of the cervix, vulva and vagina in women, penile cancer in men, and anal and oral cancer in both sexes [16]. A systemic review of the published literature revealed that 40 % of penile cancers worldwide are HPV-related [8 870-877]. 90 % of the HPV-genotypes associated with penile SCC are high-risk types, predominately caused by a single genotype accounting for 75 percent of all HPV positive cases. The most frequently found genotype in cancer is HPV 16 (72 percent of all cases) and 18 (6 %) [5].

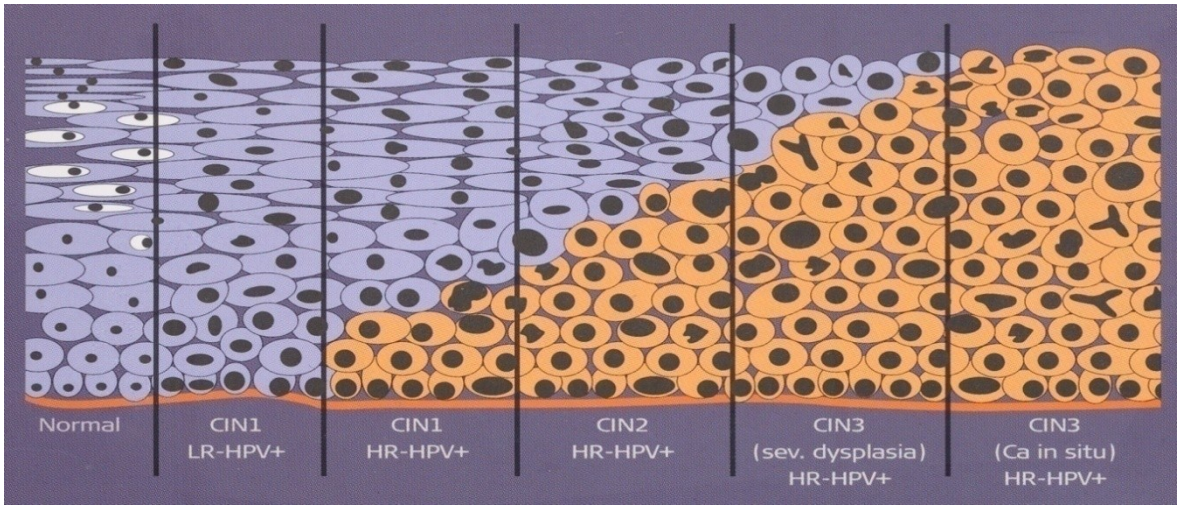
1.5.2 HPV-induced Carcinogenesis

An infection with HPV high-risk genotypes does not cause automatically a PeIN or invasive cancer. To induce an infection the virus needs to reach the basal cell layers and infiltrate the nucleus of one or more basal keratinocytes. This is more difficult in keratinized than in non-keratinized glycogenated mucosa and often requires injury or cell damage. The essential mechanism that leads to malignant transformation is the integration of the viral genome into the nucleus of the keratinocytes. In most patients the infection runs a silent course because a healthy immune system is capable of eradicating the virus within a few months or within up 2 years. Integration of the viral genome into the human DNA results in a loss of cell cycle control via binding of viral oncoproteins E6 and E7 to cell-cycle-regulatory proteins. E6 binds to p53 and E7 binds to Retinoblastoma gene (Rb). A transforming HPV-infection leads to a loss of the G1 cell-cycle control that – under physiological conditions – is regulated by a negative feedback system by the tumor suppressor proteins, particularly pRb and cyclin-depending kinase inhibitor $p16^{ink4a}$. The inhibitory function of pRb is blocked by the viral oncogene E7. Reduction of pRb activity is the suggested mechanism in proliferating cells expressing HPV high-risk E7. Through the loss of the G1 pause, the cells immediately progress into the S-phase of the cell cycle. Binding of the oncogenes induces a genetic instability that is considered to be the key in the HPV carcinogenesis. Only E7 of HPV high-risk genotypes can permanently inactivate pRb, which, in turn, leads to an over-expression of p16. E7 HPV low-risk has a lower affinity to bind pRb and has no permanent effect on the cell cycle regulation. $p16^{ink4a}$ over-expression can be detected by immunohistochemistry and serves as a surrogate marker for HPV-induced precancerous lesions and invasive cancers. Over-expression of $p16^{ink4a}$ and demonstration of HPV high-risk genotypes in a tumor allows classification of a HPV-induced carcinoma [17].

1.5.3 HPV-induced Penile Intraepithelial Neoplasia

Invasive carcinomas develop through intraepithelial non-invasive precursor lesions. In recent years, the terms carcinoma in-situ and dysplasia have been replaced by PeIN in analogy to other organs such as cervix (CIN), vulva (VIN), vagina (VAIN) and anus (AIN). Clinical terms for high-grade PeIN are Morbus Bowen, Erythroplasia de Queyrat and bowenoid Papulosis which are still used, mostly by dermatologists. HPV-induced PeIN, also referred to as classical or basaloid PeIN, is characterized by a low progression to invasive squamous cell carcinoma [6]. Just as in CIN, PeIN can be divided into PeIN I, II, or III. This depends on the extent of dysplastic involvement of the epithelium. Cytological and histopathological criteria for dysplasia are: irregular nuclear size, nuclear abnormalities, hyperchromasia, nuclear membrane irregularities, mitosis and loss of polarization. In PeIN I, the lower third of the epithelium is dysplastic, in which mitotic figures and nuclear abnormalities can be found. In PeIN II, the basal two thirds show dysplasia. In PeIN III, the entire epithelium is replaced by dysplastic epithelium by nuclear abnormalities and abnormal mitoses which can be found throughout the epithelium [18].

Figure 7: Patterns of Immunohistochemical p16 Staining (dysplastic cells are highlighted in yellow)

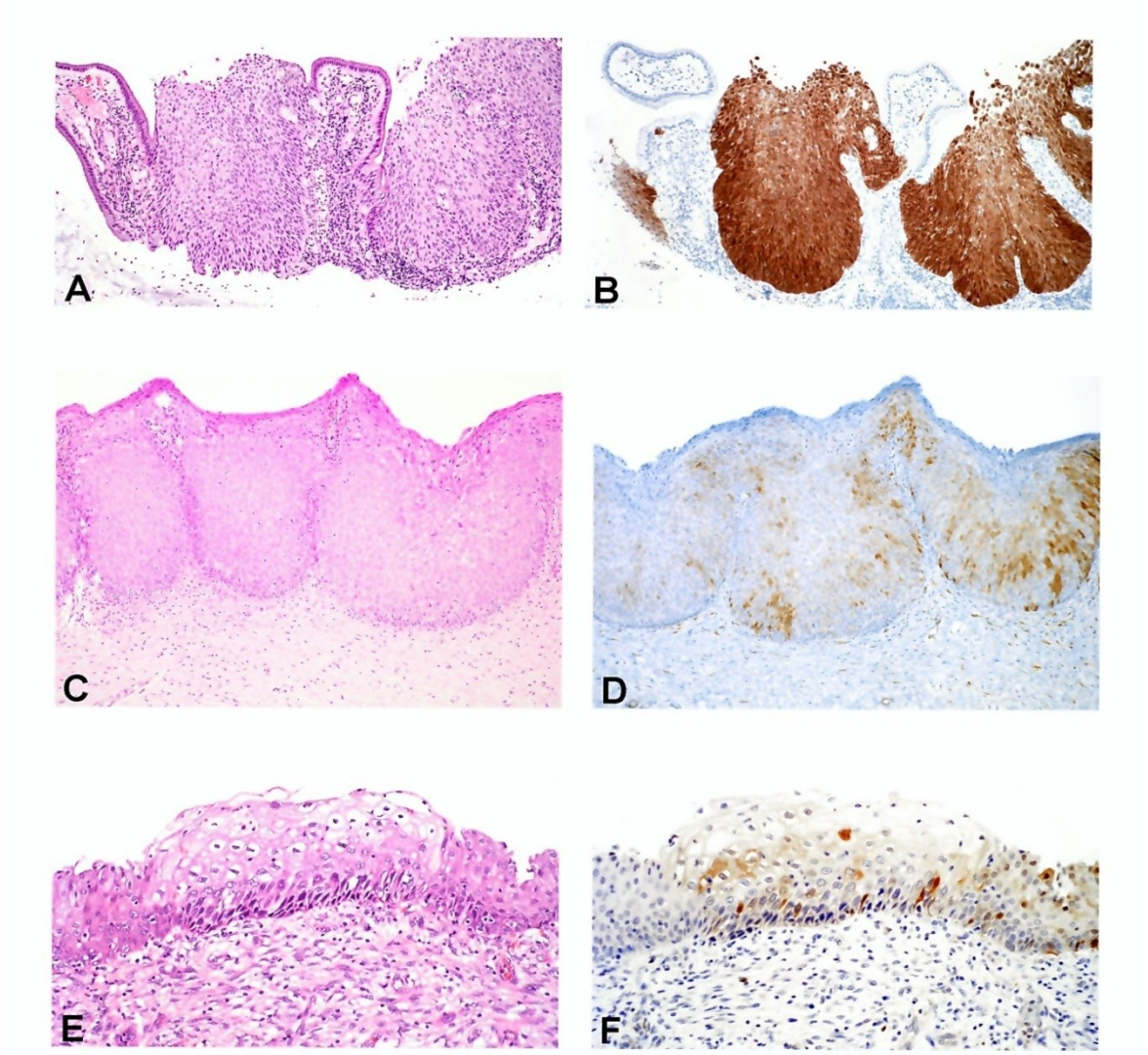


Source: CINtec^{TM} p16^{ink4a} Staining Atlas [19]

1.5.4 Correct Interpretation of p16 ^{ink4a} Staining

For a correct interpretation of over-expression, a strong continuous nuclear and cytoplasmic staining of the entire dysplastic epithelium, beginning in the basal and parabasal cells and extending continuously to the superficial layers of the dysplastic epithelium, is required. Immunohistochemical staining is divided into two expression patterns: negative including weak, focal and patchy and positive with strong and diffuse expression pattern (Fig. 7). Non-dysplastic squamous epithelia might show a weak, focal or patchy expression pattern but is normally negative for p16^{ink4a} over-expression. Only strong and diffuse staining pattern is considered as positive for p16^{ink4a} over-expression [17].

Figure 8: Examples of HPV-induced Lesions: HE Stains and their Corresponding p16 ^{ink4a} Stains

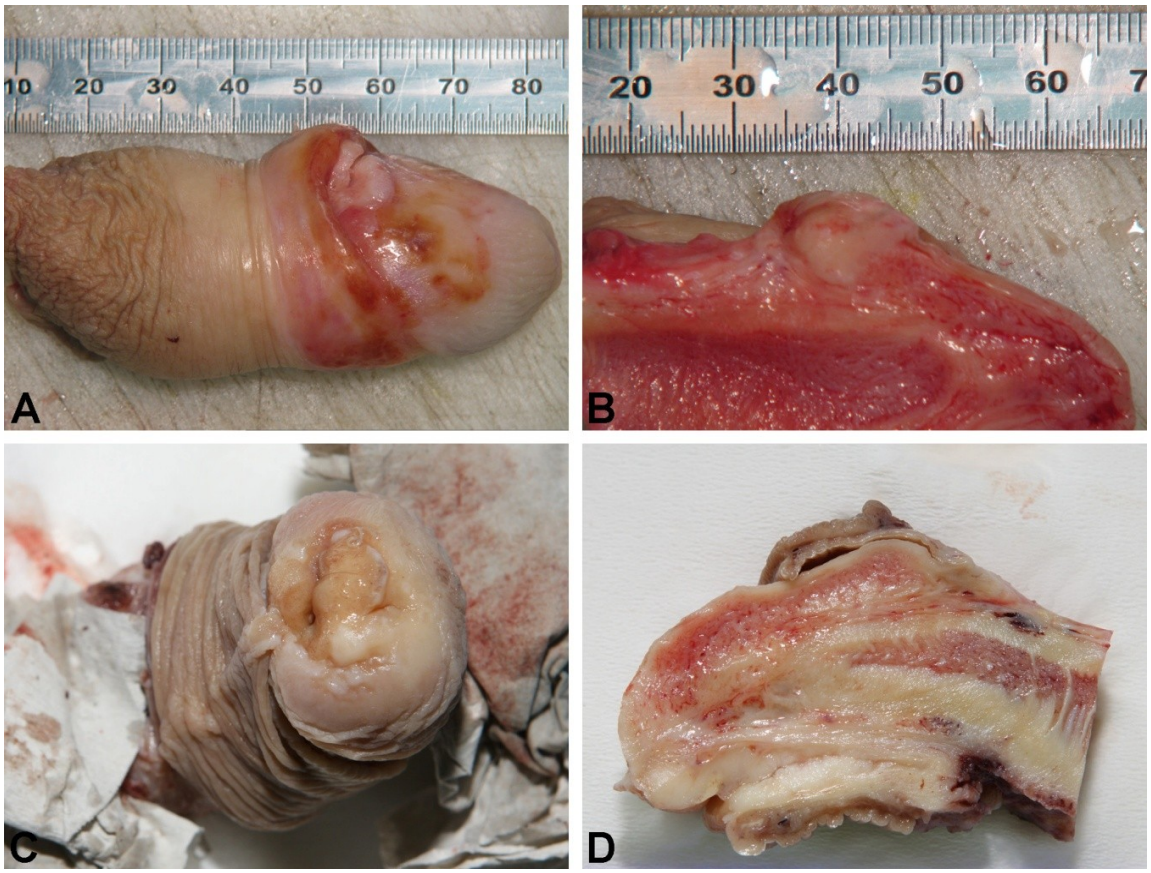


Source: Dr. Regauer, Institute of Pathology, Medical University Graz
HE stains (A, C, E) on the left side and corresponding p16 ^{ink4a} stains (B, D, F) on the opposite side. B is interpreted as positive for p16^{ink4a} over-expression (=HPV-positive), D and F are interpreted as HPV-negative
A: intraepithelial neoplasia; B: strong and diffuse p16 ^{ink4a} stain C: intraepithelial neoplasia; D: focal and patchy p16 ^{ink4a} stain E: condyloma accuminatum; F: p16 ^{ink4a} -stain with single cells

1.6 Penile Dermatoses Associated with Penile Cancer

The penis is a common site of chronic inflammatory skin diseases such as contact dermatitis, drug eruptions, psoriasis, LS and LP. They can be categorized according to the risk for developing cancer. Contact dermatitis, drug eruptions and psoriasis carry no risk for malignant transformation. The association between LS and penile cancer is well established, whereas the relationship between LP and penile cancer needs further investigations [20].

Figure 9: Carcinoma Associated with HPV High-Risk Genotypes

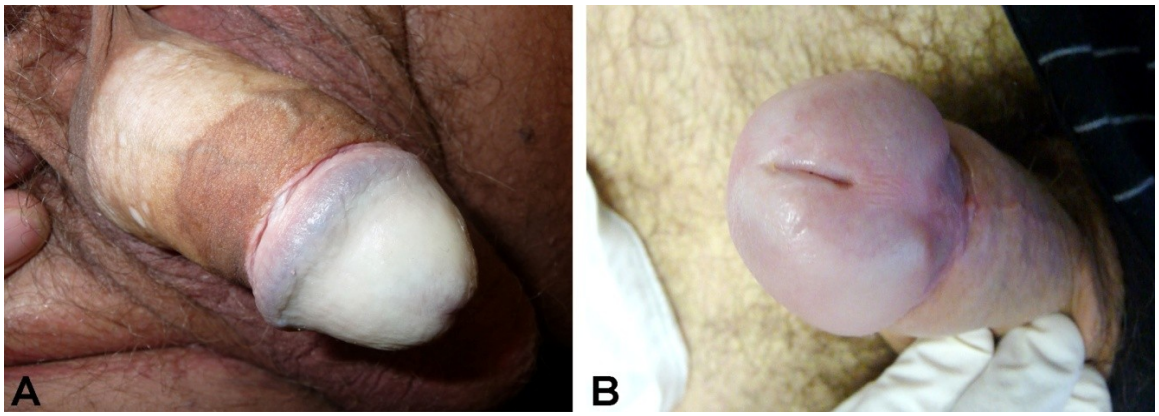


Source: Dr. Regauer, Institute of Pathology, Medical University Graz
HPV-associated penile cancers: A: penectomy specimen showing carcinoma in sulcus coronarius B: cross section of carcinoma shown in A, C: penectomy specimen showing HPV16-positive, pT3 carcinoma in the glans penis and D: cross section of carcinoma shown in C

1.6.1 Lichen Sclerosus

LS is a lymphocyte-mediated, chronic, relapsing, non-contagious inflammatory disease affecting the skin, in particular the anogenital region. Clinically, the initial presentation of LS is whitish, porcelain-like sclerotic plaques. Predilection sites in women are vulva, perineum and perianal skin and in men glans penis and the foreskin. Disease activity determines clinical course and disease progression [21]. The extragenital region is affected rarely. LS involves the glans penis and prepuce, sometimes causing progressive phimosis. Occasionally, the meatus can be affected as well. The involvement of the meatus and urethra has been described to vary from 2% to 40% and depends on the duration of the disease and the activity of LS. The prepuce can be shortened (frenulum breve) as a consequence of the sclerotic process. However, involvement of the urethra by LS is a rare complication and only found in longstanding and untreated diseases. LS is responsible for 80% to 90% of acquired phimosis. Thus, any case of secondary phimosis should be considered suspicious for LS [21].

Figure 10: Clinical Variants of Lichen Sclerosus



Source: Dr. Razmara, Department of Urology, Medical University Graz
A: Penis with vitiligo after circumcision for LS; B: Glans penis with LS-related changes around the meatus uethrae after circumcision

Terminology and Prevalence

In 1887, LS was described by Hallopeau for the first time [22]. Since then several terms have been used for LS: ichthyosis, leucoplakia, kraurosis, lichen sclerosus et atrophicus, white spot disease, dystrophy [23] and balanitis xerotica obliterans [24]. These outdated terms are synonymous for end stage LS but are not useful in describing an early stage of the disease. Therefore, the World Health Organization together with the International Society for the Study of Vulvovaginal Disease (ISSVD) made the following recommendation: LS is the preferred term for all disease stages. In addition, disease activity and stage has to be added to diagnosis [25]. The prevalence of LS is not exactly known. Former studies report that the number of women suffering from LS is higher than that in men but current studies considered LS-prevalence as being equally common in both sexes [22]. The true overall-prevalence of LS is most likely higher than expected and accounts for up to 8% in gynecology practices. Penile dermatoses of the prepuce and the penis are underappreciated although a high estimated number of unknown cases are discussed [26].

Etiology

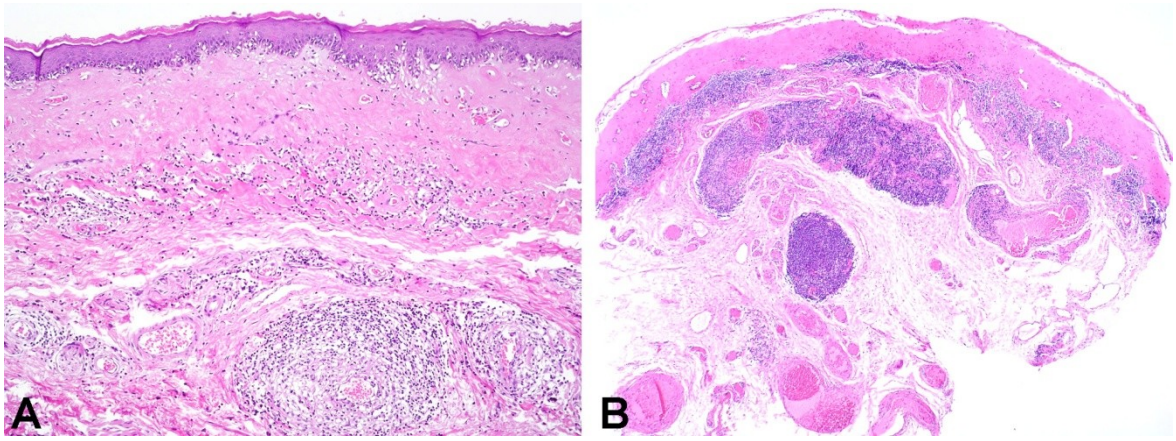
The exact etiology of LS is unknown. Discussed are immune dysregulation or autoimmune mechanism(s) with a multifactorial background [23], in analogy to psoriasis. Additionally, genetic factors such as familial clustering and an association with special HLA-class II-antigens have been proposed. Borrelia burgdorferi infections after tick bite have been observed with initiation of LS. Abnormal enzyme metabolism, particulary atrophy of elastin fibers, lowered estrogen level or abnormal testosterone metabolism are out-dated theories. Elastin fibers are present in late stages of the disease [26]. Nowadays, LS is considered to be the result of an immune response to a putative antigen, which may be a viral/bacterial anti-

gen or endogenous substances. Most likely is a mechanism similar to psoriasis, in which an immune response is launched in response to a bacterial infection [27]. In this context it should be pointed out that patients with LS suffer simultaneously from psoriasis [23]. In case of plaque-psoriasis, T-lymphocytes recognize a protein sequence found in cell walls of group A Streptococcus and in keratinocytes [28]. These activated T-lymphocytes cannot distinguish between foreign and body's own cells [23]. Many patients with LS suffer from concomitant autoimmune diseases such as alopecia areata, vitiligo and Hashimoto Thyroiditis and pernicious anemia being diseases characterized by disease-related or organ-related antibodies [23]. So far, no disease-related antibodies could be detected for LS. Antibodies against extracellular matrix from blood samples of LS-patients are considered as secondary phenomenon after excessive destruction [23].

Immune Dysregulation-Inflammatory Infiltration-Disease Activity

The immune dysregulation in LS is evidenced by presence of numerous inflammatory cells found in the skin along the basal membrane (so-called lichenoid reaction pattern). The extent of lymphocytic infiltrate, consisting of T-, B-lymphocytes and antigen-presenting dendritic cells, determines the disease activity. The activated lymphocytes produce cytokines such as interleukin 4, 6, tumor necrosis factor β and γ, which are responsible for development of fibrosis and sclerosis. Through longstanding antigen contact, the immune system produces antigen-specific T-cell clones [23]. Molecular analysis for "monoclonal rearrangement of gamma locus of T-cell receptor" allows the detection of antigen-specific T-lymphocytes. The amount of monoclonal T-lymphocytes ranges from 1.4 % to 21% in the lymphocytic infiltrate [29]. In active LS, a vasculitis, e.g. an inflammation of vessel walls, can be observed. In LS, two major types of vasculitis can be distinguished: the lymphocytic vasculitis and the leukocytoclastic vasculitis. The lymphocytic vasculitits can be further divided into a) the lymphohistiocytic vasculitis with concentrically arranged lymphocytes and histiocytes infiltrating and damaging the outer venous vessel walls and occasional localized accumulation of histiocytes (the so-called granulomas vasculitis) and b) a pure lymphocytic vasculitis with densely clustered lymphocytes around thin-walled vessels, fibrin precipitate within the vessel walls. The lymphocytic vasculitis can also occur in large muscular blood vessels. The leukocytoclastic vasculitis with complete fibrinoid destruction (=necrosis) of the vessel walls with infiltration of neutrophilic granulocytes is rare in LS. In the vascular lumen fibrin emboli can be found [30]

Figure 11: Variants of Vasculitis in LS

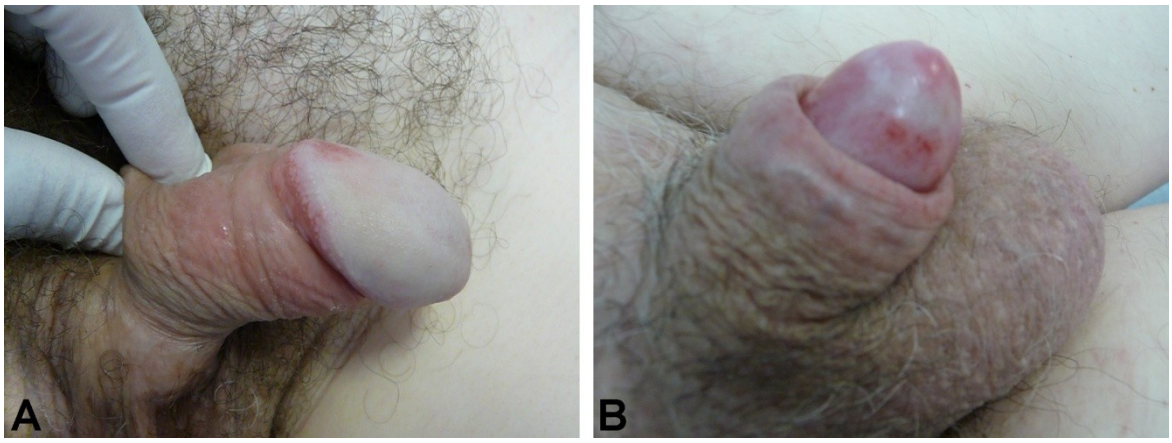


Source: Dr. Regauer, Institute of Pathology, Medical University Graz
A: lymphohistioytic vasculitis with granulomatous accumulation of histiocytes; B: lymphocytic vasculitis with dense accumulation of lymphocytes in advanced LS with degenerative surface changes

1.6.2 Lichen Planus

LP is a lymphocyte-mediated, chronic, relapsing, non-contagious inflammatory disease affecting the skin and mucosa. The overall prevalence worldwide is less than 1 percent. A similar prevalence has been reported in Austria. LP is a disease with no sex predilection, but it is rarely reported in childhood [31]. LP presents with several clinical variants. It begins commonly at the flexor aspect of the wrists, the forearms, the extensor aspect of the hands and ankles. In one third of patients LP generalizes within 3 months to the rest of the body. LP can affect hair follicles and nails. Many patients suffer from oral involvement. A sole oral manifestation without developing skin lesions is estimated to account for up to 35%. Other affected mucous membranes are those of the larynx, oesophagus, nose, anus and genitalia. Genital involvement occurs in at least 25% of patients. Clinically, lesion of LP represents as smooth, shiny, flat-toped polygonal papules ranging from several mm to 1 cm in diameter. Early lesions are characterized by a red or violaceous color. Older lesions are white and are characterized by a slightly scaly surface. In mucosal sites, LP is often erosive and Wickham's striae, e.g. fine, thin white lines usually crossing and / or surrounding the papules, are typical findings. According to the configuration of lesions, LP can be described as annular or linear. Annular LP is more common in the anogenital region, in particular on the scrotum and penis. LP affecting the foreskin and glans penis may produce a phimosis. Morphologically, the following types of LP can be distinguished: hypertrophic or verrucous LP, atrophic LP, vesiculobullous LP, erosive and ulcerative LP. Special forms of LP include drug-induced LP [31]. Fewer than 100 cases of familial LP have been reported. These cases tend to represent clinically as erosive or ulcerative lesions and are more common in young adults and children [31].

Figure 12: Clinical Pictures of LP



Source: Dr. Razmara, Department of Urology, Medical University Graz
A: Penis after circumcision for LP; B: Erosive LP of glans penis after circumcision

Etiology and Immune Dysregulation

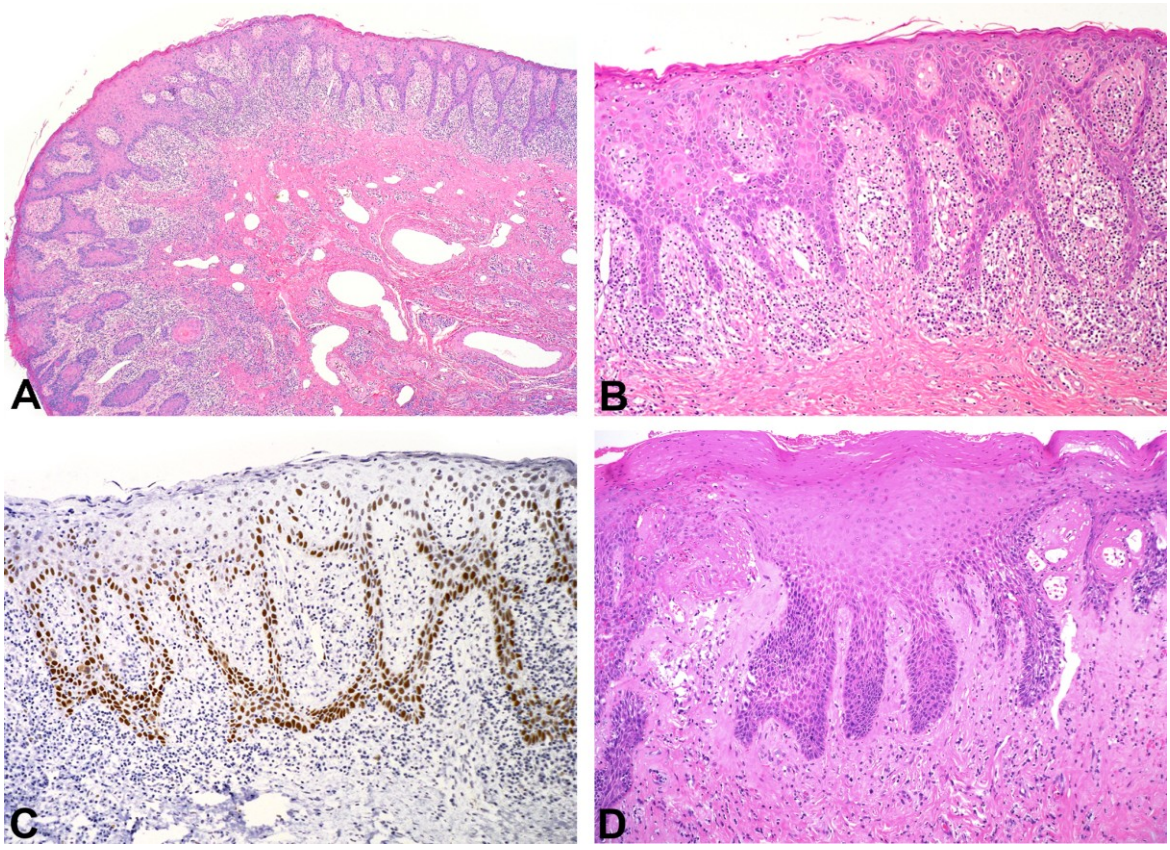
The exact etiology of LP remains unclear. An association with bacterial and viral infections and autoimmune diseases, in particular autoimmune chronic active hepatitis, primary biliary cirrhosis and postviral chronic active hepatitis has been described. Other causes, e.g. metabolic, psychosomatic and genetic causes similar to LS have been discussed [32]. In analogy to LS, there is good evidence for an immunologic dysregulation [31]. Current findings postulate that LP represents an abnormal delayed hypersensitivity reaction to an antigen of possible epidermal origin [32]. In skin lesions of LP, the lymphocytic infiltrate consists of CD4+ and CD8+ T-cells. In contrast to LS, pro- and anti-inflammatory cytokines are activated simultaneously in LP thus determining disease activity and clinical behavior. Combined effects of cytokine and lymphotoxin release, tumor necrosis factor β and the presence of cytotoxic T- cells, result in destruction of basement membrane and basal keratinocyte death (apoptosis). Similar to LS, activated CD8+ lymphocytes show clonal expansion. The role of LP in penile cancer so far has received inadequate attention thus malignant transformation in LP is not well documented [29,33].

1.6.3 HPV-negative differentiated Penile Intraepithelial Neoplasia

Invasive HPV-negative carcinomas develop through intraepithelial non-invasive precursor lesions, termed d-PeIN. d-PeIN are known to arise in the background of LS but their association with LP is less well established. Just as differentiated VIN in vulvar cancer, d-PeIN is mostly detected in surgical specimens adjacent to HPV-negative invasive penile SCC, and d-PeIN is rare in its isolated form without SCC. d-PeIN is considered a high-grade lesion with rapid progression, often in less than 6 months to invasive SCC [5]. Histologically, d-PeIN is characterized by acanthotic epithelium with premature keratinization, elon-

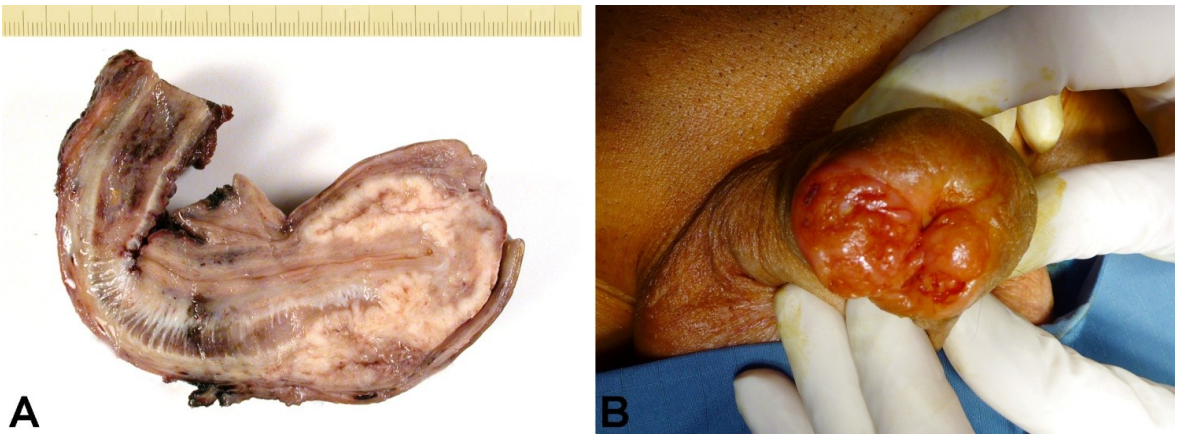
gated rete ridges, mitoses and nuclear atypia in basal keratinocytes, but typically normal superficial keratinization. Other frequent findings are keratin pearl formation and prominent intercellular bridges. HPV-related cell changes, e.g. koilocytosis, are absent in d-PeIN (Figure 13). Immunohistochemically, d-VIN and d-PeIN are negative for $p16^{INK4a}$ overexpression but mostly positive for p53 staining.

Figure 13: HE and Immunohistochemical Stains of d-VIN and d-PeIN



A: Low-power view HE stain of keratinizing d-VIN; B: HE stain of keratinizing d-VIN with nuclear atypia, hyperchomasia and mitotic atypia of basal keratinocytes in enlonged rete ridges C: Corresponding p53 stain of B, Source: Regauer from the Journal Histopathology [34]
D: d-PeIN; Source: Dr. Regauer, Institute of Pathology, Medical University Graz

Figure 14: Penile Cancers Associated with Dermatoses



A: penectomy specimen with white to grayish tumor tissue infiltrating the corpus spongiosum and cavernosum, and urethra. Source: Dr. Regauer, Institute of Pathology, Medical University Graz
B: Penile cancer of the foreskin arising in the background of LP Source: Dr. Razmara, Department of Urology, Medical University Graz

1.7 TNM Classification for pT1 Penile Carcinomas

Penile cancer can be classified according to TNM classification system allowing the division of primary penile cancers depending on the extent of invasion in pT1, pT2, pT3 and pT4 tumors (Fig.15). Correct prediction of lymph node metastases - particularly in early stage pT1 penile cancers – is difficult. Overall, only 50% of patients with penile cancer will have metastases. There is evidence that 75% of prophylactic inguinal lymphadenectomies performed in pT1 penile cancers are unnecessary, but patients suffer from long-term morbidity of wound healing complications, erysipelas and lymph edema [35]. Clinically, patients are divided into two groups: palpable and impalpable, clinically negative regional lymph nodes. The need for prophylactic inguinal lymphadenectomies in patients with impalpable lymph nodes is controversially discussed [36-39]. Several histopathological staging proposals have been developed for correct prediction of lymph node metastases, but the TNM classification is the most widely used classification. Tumor grade and lymph vessel invasion are independent prognostic factors for occult nodal involvement. The latest TNM classification of 2009 divides pT1 penile SCC according to the histological grade and present/absent lymphatic invasion. PT1a carcinomas represent histological grade G1 or G2, and absent lymphatic invasion. PT1b Carcinomas represent with either G3, G4 and/or show lymphatic invasion [40]. In clinically node-negative patients the European Association of Urology (EAU) considers pT1 penile cancers with low tumor grade as low-risk for lymph node metastases [38] and histological grade G2 as intermediate risk for occult inguinal LN metastases [41]. The EAU recommends the micro-invasive technique of sentinel lymph

node (SLN) identification in penile cancer ≥pT1G2 [38]. In case, this technique is not available, the decision for a prophylactic lymphadenectomy should be based on assessment of additional histological risk factors, e.g. histological subtypes, depth of invasion [3,42,43] and perineural invasion [44,45].

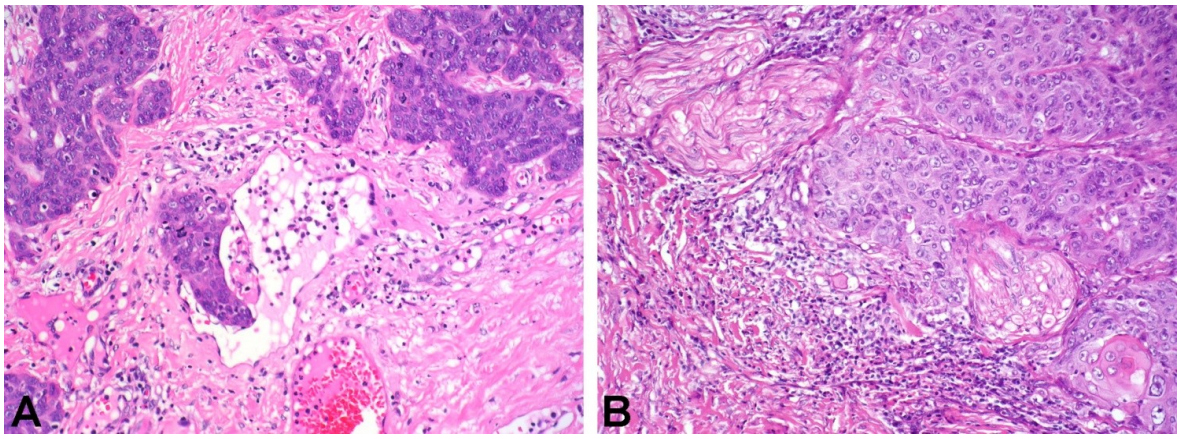
Figure 15: TNM Clinical Classification

T – Primary Tumour

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Ta	Non-invasive verrucous carcinoma[1]
T1	Tumour invades subepithelial connective tissue
T1a	Tumour invades subepithelial connective tissue without lymphovascular invasion and is not poorly differentiated or undifferentiated
T1b	Tumour invades subepithelial connective tissue with lymphovascular invasion or is poorly differentiated or undifferentiated
T2	Tumour invades corpus spongiosum or cavernosum
T3	Tumour invades urethra
T4	Tumour invades other adjacent structures

Note: Verrucous carcinoma not associated with destructive invasion
Source: TNM Classification of Malignant Tumours [40]

Figure 16: Histopathological Prognostic Factors



Source: Dr. Regauer, Institute of Pathology, Medical University Graz
A: lymphatic invasion in basaloid SCC B: perineural invasion in basaloid SCC

2 Material and Methods

2.1 Clinicopathological Data

During the past 25 years, 164 penile cancers were diagnosed at the Institute of Pathology, Medical University Graz, Styria, Austria. Tissue blocks of all penile cancers were retrieved from the archives of the Institute of Pathology, Medical University Graz, in the State of Styria, Austria. For 8 patients no tissue blocks or no tumor tissue in the blocks was left for analysis, in 6 cases DNA quality was insufficient for analysis. In 40 circumcision specimens, 24 excisions of glans penis and 86 partial/total penectomies (patient's age range 16-89 years), 29 PeINs, 6 d-PeINs, 109 SCCs and 6 clear cell carcinomas (5 of which were previously published [9]) were diagnosed. Invasive cancers were classified according to the 2009 TNM classifications system [46] and histologically subtyped [6,42]. The presence of peritumoral LS, LP, precursor lesions and inflammatory infiltrate was recorded.

2.2 HPV Detection

Genotyping was performed on formalin-fixed and paraffin embedded tumor tissue. After DNA extraction with the QIAamp DNA Mini Kit (Quiagen No: 51304), according to manufacturer's protocol HPV-genotyping was performed with INNO-LiPA HPV GENOTYPING EXTRA (Innogenetics Diagnostic, Germany). In brief, the INNO-LiPA HPV Genotyping Extra – Kit is based on the principle of reverse hybridization. The SPF10 primer set amplifies a 65-bp region in the L1 open reading frame of the HPV genome. To monitor sample quality, an additional primer pair for the amplification of the human HLA-DPB1 gene was added. After PCR, the resulting biotinylated amplicons were denatured and hybridized with specific oligonucleotide probes. All probes were immobilized as parallel lines on membrane strips. Hybridization was followed by stringent washing. Streptavidin-conjugated alkaline phosphatase was added, which binds to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen (substrate) yields a purple precipitate.

On a single strip 28 sequence-specific HPV DNA probe lines and 4 control lines were fixed. Interpretation of the signal pattern allowed the detection of HPV-low-risk 6, 11, 40, 43, 44, 54, 70, HPV-high-risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 genotypes, probable high-risk 26, 53 and 66 genotypes and HPV 69, 71 and 74, which are not classified as high-risk, but as probable high risk or low risk genotypes. All HPV genotypes

for which the type-specific line pattern was a subset of the full line pattern observed on the strip had to be scored as present or possibly present in the sample. An HPV type was possibly present, when all probe lines forming its specific hybridization pattern were already part of a single or multiple specific hybridization patterns of other HPV types. The genotypes presenting possible co-infections are reported in brackets, e.g. HPV18-(HPV39) [47].

2.3 Immunohistochemical Analysis

Immunohistochemical demonstration of over-expression with antibody to $p16^{INK4a}$ (mtm laboratories, Heidelberg, Germany, CINtec) has emerged as "surrogate marker" for transforming infection with HPV high-risk genotypes which requires integration of the viral genome into the human genome. Over-expression is absent in tissues infected with HPV low-risk genotypes or in infections with HPV high-risk genotypes in the absence of integration and transformation. Only diffuse and strong staining in the entire dysplastic epithelium or invasive SCC was interpreted as positive. Focal, patchy, weak immunohistochemical staining was defined as negative. d-PeIN was stained with antibody to p53 (DAKO Denmark).

2.4 Detection of Rearrangements of the T-cell Receptor Gamma Locus

Only cases with dense T-cell dominant inflammatory infiltrates, e.g. 10 dermatoses associated SCCs, 2 HPV-induced PeINs in patients with underlying LS and LP and 1 d-PeIN were subjected to PCR analysis for rearranged T-cell receptor gamma locus. Genomic DNA was extracted from formalin-fixed, paraffin embedded tissue blocks as described previously. PCR analysis was performed using the *TCRG* Gene Clonality Assay kit (InVivoScribe Technologies, San Diego, CA) based on the BIOMED-2 studies. Briefly, genomic DNA was used as a template for PCR with consensus primers for the variable (V1-11) and the joining regions of the T-cell receptor gamma locus. The presence of amplifiable DNA was assessed using the control DNA and the primer set from InVivoScribe Technologies. The PCR products were separated by capillary electrophoresis using an automated sequencing system, ABI310 (Applied Biosystems Invitrogen, Foster City, CA), and analyzed using Genescan software (Applied Biosystems Invitrogen) [48].

2.5 Prognostic Correlation of 76 pT1 Penile Cancers

The clinical follow-up of 76 pT1 penile cancers was median 47 months (range 5-265 months) defined as period from diagnosis until the most recent follow-up or death. Primary penile cancers were reclassified according to the 2009 TNM classification [46] based on a

combined score of tumor grade and lymphatic invasion (pT1a = G1, G2, no lymphatic invasion; pT1b = lymphatic invasion and/or G3, G4). The vast majority of 72/76 pT1 penile cancers were SCCs, with grades G1-4. Was more than one grade present, the highest grade was used for reclassification independent of the percentage of tumor volume. Only 4/76 penile cancers were clear cell carcinomas. Presence of lymph node metastases and survival were correlated with the subclassification of the 2009 TNM classification, with perineural invasion, growth patterns *(superficial, papillary and verrucous)* and depth of invasion (*≤5mm, 6-14mm, ≥15mm)*. Lymphovascular and perineural invasion at the tumor front were evaluated on hematoxylin and eosin (HE) stained sections routinely used in daily surgical pathology practice. For evaluation of possibly missed lymphatic invasion on routine HE stained sections, a retrospective immunohistochemical analysis of all metastatic pT1 cancers was performed with antibody to D2-40 (Dako, Denmark), a marker labeling exclusively lymphatic vessels.

Etiologically, the pT1 penile cancers were divided into HPV-negative carcinomas, which arose in the background of LS and LP and HPV-induced carcinomas.

Statistical analysis was performed by means of SPSS 11.0 for Windows software package (SPSS Inc., Chicago, USA). Pair wise associations between the variables were tested with chi-square test or Fisher's exact test. Kaplan-Meier plots and log rank test were employed for survival analysis.

3 Results

3.1 Bimodal Pathway in Penile Carcinogenesis in Styria

Evaluated were 115 invasive carcinomas (TNM 2009: 88pT1, 16pT2, 11pT3; Fig.17) diagnosed during the past 25 years. Additionally evaluated were isolated lesions of 29 PeINs and 6 d-PeINs diagnosed during the past 3 years. PeIN was identified in 27 circumcision specimens and 2 excisions of glans penis. d-PeIN was identified in 4 circumcision specimens, in 1 glans penis excision and 1 distal penectomy. Invasive cancers (28 well differentiated keratinizing SCCs, 10 papillary SCCs, 11 verrucous SCCs, 58 non-keratinizing / basaloid SCCs, 1 condylomatous SCC, 1 spindle cell SCC and 6 clear cell carcinomas) were found in 5 circumcision specimens, 18 glans penis excisions and 86 penectomies. Average age of patients with PeIN was 64 years (range 16-83 years) and of patients with invasive cancer 66 years (range 18-89 years).

3.1.1 HPV-induced Invasive Carcinoma (see Table 1)

63 invasive SCCs (54pT1, 7pT2, 2pT3; 13 poorly differentiated keratinizing SCCs, 45 basaloid SCCs, 3 verrucous, 1 condylomatous, 1 spindle cell SCC) and all 6 clear cell carcinomas (4pT1, 2 pT3) carried HPV high-risk genotypes. HPV high-risk 16 as single genotype (Fig.17 strip 6) was detected in 42/54 pT1, 4/7pT2, 2/2pT3 SCCs and all 6 clear cell carcinomas, HPV-high-risk 45 as single genotype in 2/62 SCCs (Fig.17 strip 2). Type-specific HPV high-risk 33 with bands indicating a possible co-infection with HPV high-risk 52 and HPV low-risk 54 was detected in 5/63 SCCs (4pT1, 1pT2) and HPV high-risk 18 in 1/63 SCC (pT1). Multiple type-specific HPV genotypes in addition to HPV high-risk 16 were observed in 2/63 SCCs. Multiple type-specific HPV high-risk genotypes without HPV high-risk 16/18 were detected in 4/63 SCCs (Fig.17 strips 3 and 4). Co-infections with low- and high-risk HPV genotypes were seen in only 1 SCC. HPV low-risk genotype was detected in the condylomatous SCC of the penis shaft (Fig.18 a and b) with demonstration of HPV low-risk 6 as single genotype (Fig.17 strip 1). Peritumoral p16- and HPV-positive PeIN was identified in all invasive SCCs and clear cell carcinomas. Three men with HPV high-risk 16 positive SCCs had also LS (1) or LP (2).

Table 1: HPV-genotyping Results of 150 Penile Cancers and Precursor Lesions

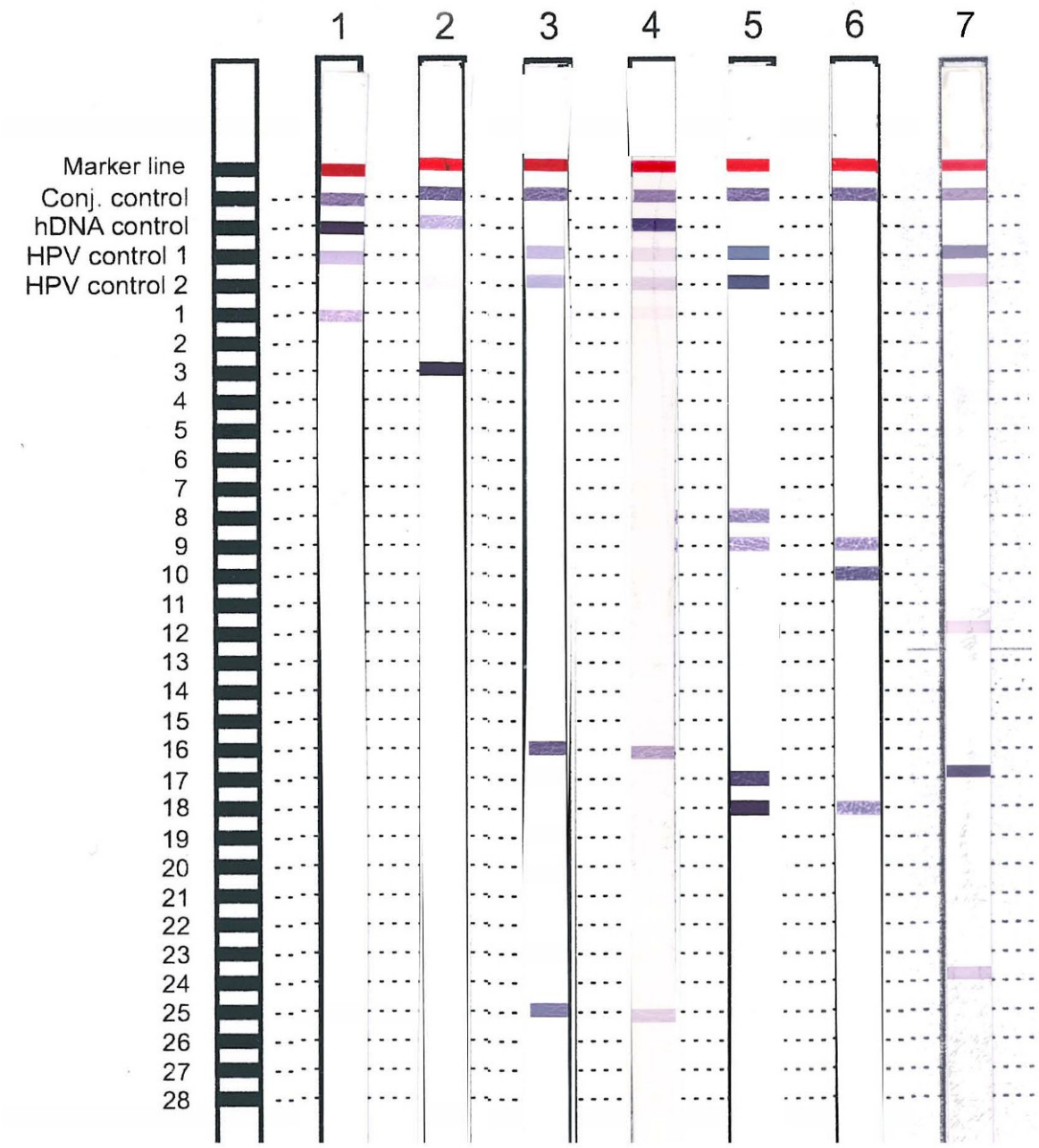
	Total	d-PeIN	PeIN	pT1	pT2	pT3
Total	150	6	29	88	16	11
HPV negative	52	6	0	30	9	7
HPV positive	99	0	29	58	7	4
HPV high-risk-genotypes	98		29	58	5	4
Single type-specific HPV HR 16	76		22	46	4	4
Single type-specific HPV HR 18	1			1		
Single type-specific HPV HR 45	2			1*	1	
Total single HPV-HR genotype	79		22	48	5	4
Type-specific HPV-HR genotypes with possible coinfection						
HPV HR16 (HR31, 33, 52, 53, 58) (LR 39)	1		1			
HPV HR 18 (HR 39)	1		1			
HPV HR 33 (HR 52, LR 54)	6		1	4	1	
HPV HR 73 (HR 39, HR 68)	1		1			
Coinfection HPV-HR genotypes						
HPV HR 18, 74†(HR 39)	1		1			
HPV HR18, HR 51, pHR 66‡(HR 39)	1		1			
HPV HR 16, HR 31 (HR 52, LR 54)	1			1		
HPV HR 16, HR 52	1			1		
HPV HR 31, HR 73 (HR 39, HR 52, HR 68, LR 54)	1			1		
HPV HR 31, HR 51 (HR 52, LR 54)	1			1		
HPV HR 31, HR 33, HR 58 (LR 40, LR 54)	1		1			
HPV HR 45 and LR 6	1			1		
HPV 69/71†LR 54 (HPV33)	1			1		
HPV low-risk, genotype single HPV LR 6	1				1*	
HPV-HR genotypes different from HPV HR 16/18	20		7	11	2	0

*p-16 negative squamous cell carcinoma.
†Not classified as HR, probable HR, or LR genotypes, genotypes in brackets present possible coinfections, according to Munoz et al.[17]
‡Considered probable high-risk (pHR) genotypes.
HR indicates high risk.

3.1.2 HPV-induced Penile Intraepithelial Neoplasia (see Table 1)

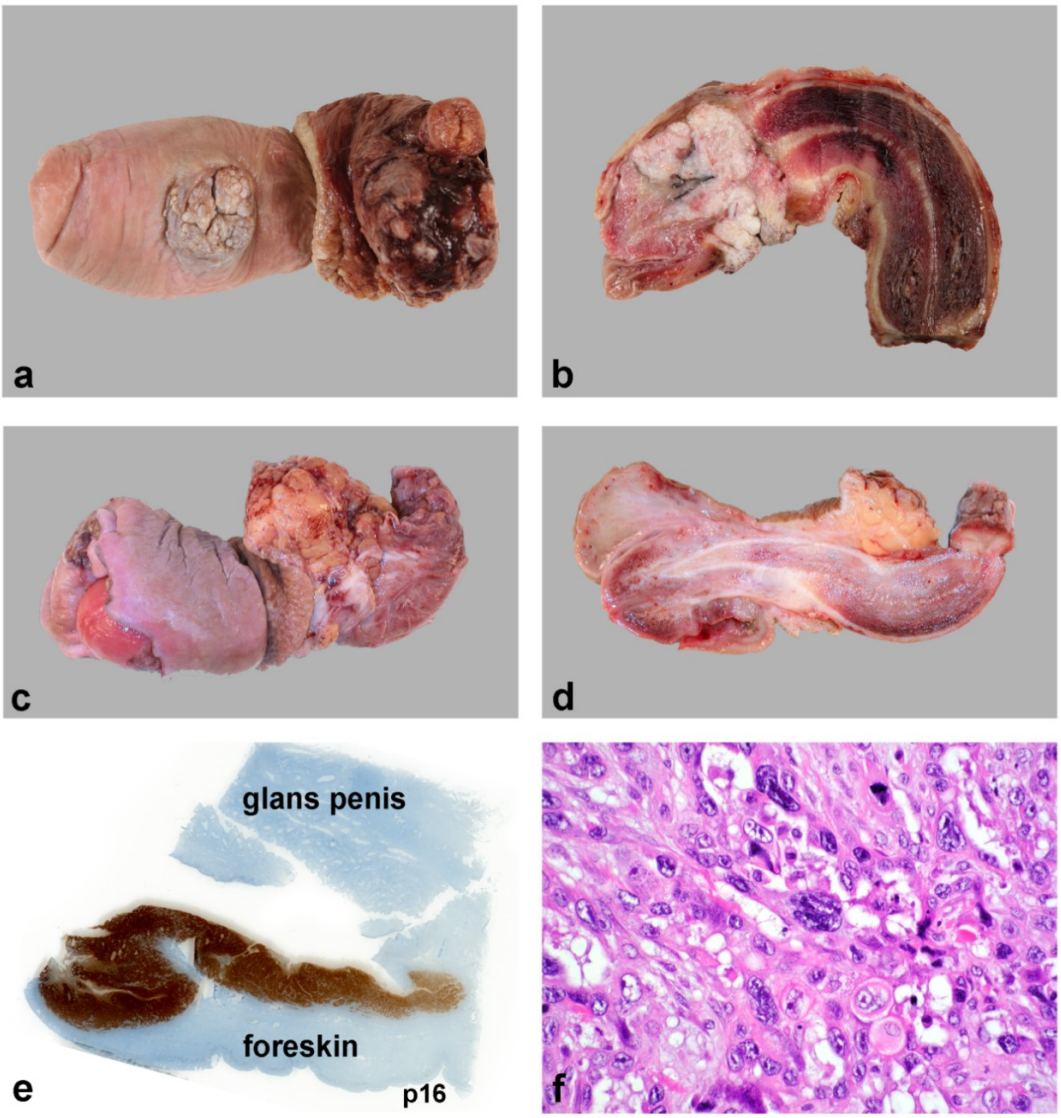
All 29 PeINs harbored HPV high-risk genotypes. HPV high-risk 16 was observed as single genotype in 22/29 PeINs. Type-specific reaction products for HPV high-risk 16, HPV high-risk 18, HPV high-risk 33 and HPV high-risk 73 in addition to non-type-specific reaction products indicating a possible co-infection with other HPV genotypes was identified in 4 PeINs. Two PeINs revealed multiple type-specific HPV genotypes including HPV high-risk18 (Fig.17 strip 5). Only 1 PeIN contained multiple type-specific HPV high-risk genotypes other than HPV16/18. Three of 29 PeINs were incidental discoveries in circumcision specimens for phimosis, 2/29 patients with PeINs also suffered from LS and LP. All 29 PeINs and 61/63 HPV high-risk genotype positive invasive cancers showed $p16^{INK4a}$ overexpression (Fig.18e). One SCC of glans penis with HPV-high-risk-45-positivity was repeatedly $p16^{INK4a}$-negative (Fig.17 strip 1). No staining with antibody to $p16^{INK4a}$ was observed in the HPV low-risk 6 condylomatous SCC of the penis shaft (Fig. 18c), in 6 d-PeINs and 46 invasive HPV-negative SCCs.

Figure 17: Strips after Genotyping



Strip 1 HPV low-risk 6 single genotype detected in specimen Fig.18a/b
Strip 2 HPV high-risk 45 single genotype
Strip 3 HPV high-risk 33 (high-risk 52, low-risk 54). Type-specific HPV high-risk 33 genotype was identified with non-type-specific reaction products indicating a possible co-infection with HPV high-risk 52, HPV low-risk 54.
Strip 4 HPV high-risk 31 and HPV high-risk 51 (high-risk 52, low-risk 54). Type-specific HPV high-risk 31 and HPV high-risk 51 genotypes were identified with non-type-specific reaction products indicating a possible co-infection with HPV high-risk 52, HPV low-risk 54.
Strip 5 HPV high-risk 18 and probable HPV high-risk or HPV low-risk 74 (HPV high-risk39). Type-specific HPV high-risk 18 and probable HPV high-risk or HPV low-risk 74 genotypes were identified with non-type-specific reaction products indicating a possible co-infection with HPV high-risk 39.
Strip 6 HPV high-risk 16 single genotype detected in specimen Fig.18c-f

Figure 18: Penectomy Specimens, HE stains and Immunohistochemical Stains of HPV-positive Squamous Cell Carcinoma



a) A p16 ^{INK4a} -negative, HPV low-risk 6 positive condylomatous SCC (pT3) arising in the skin of penis shaft in a 59-year-old patient
b) Cross section of the penis revealed an infiltration through the entire penis shaft and urethra.
c) Penectomy specimen of a 52-year-old patient with an ulcerated SCC (pT2) involving the foreskin and glans penis.
d) Cross section of the penis showed extensive SCC predominantly involving the inner side of the fore-skin,
e) The carcinoma showed p16 ^{INK4a} over-expression (genotyping revealed HPV high-risk16 as the single genotype) and
f) Corresponded to a poorly / undifferentiated SCC.

3.1.3 HPV-negative differentiated Penile Intraepithelial Neoplasias and Squamous Cell Carcinomas

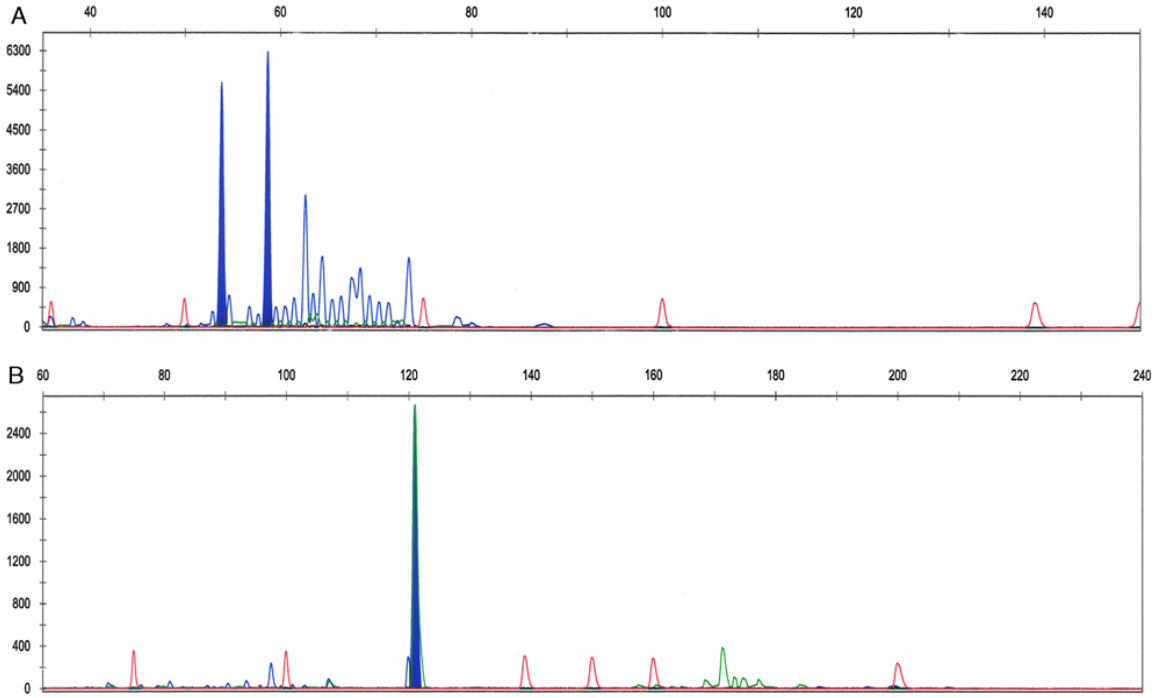
Among the 46 HPV- and p16 ^{INK4a} -negative invasive SCCs (30/88 pT1, 9/16 pT2 and 7/11 pT3, average age 65 years, range 35-91 years) were 28 highly to moderately keratinized SCCs, 10 papillary SCCs and 8 verrucous SCCs. Isolated lesions of 6 d-PeINs were also HPV- and p16 ^{INK4a} -negative. In some patients LS and LP were clinically visibly (Fig. 20A a-d). Sixteen of 46 HPV-negative SCCs were pT2/pT3 cancers, e.g. they accounted for the majority in these tumor stages despite a histological grade of G1 and G2 (Fig.20B e). Archival tissue blocks of 11/46 SCCs did not contain enough peritumoral tissue for evaluation of the presence of LS and LP. Histological features of advanced LS with flat atrophic or hypertrophic epithelium with hyperkeratosis, diffuse basement membrane thickening and a broad band-like submucosal sclerosis with a lymphohistiocytic infiltrate below the sclerosis were identified in 26 SCCs. In 3 SCCs classical features of fully developed active LP with wedge shaped hypergranulosis, saw-tooth-like elongation of rete ridges with apoptotic basal keratinocytes, were observed. Six SCCs showed atrophic flat epithelium with hyperkeratosis, accentuated stratum granulosum and occasional basal keratinocyte apoptosis with focal sub epithelial sclerosus (Fig.20B f). Peritumoral d-PeIN with elongated rete ridges, premature squamatization, p53 positive keratinocyte nuclei, basal keratinocyte atypia and mitotic activity was present in 11 LS-associated SCCs and in 7 LP-associated SCCs (Fig. 20B g/h). Three of 6 isolated d-PeINs were solitary verrucous plaques of 1-2 cm diameter in patients with LP. Three of 6 d-PeINs were identified incidentally and multifocally during histological work-up of specimens with focal hyperkeratotic areas within lesions of LP (1; Fig. 20B e/f) or LS (2). d-PeIN independent of chronic inflammatory skin diseases was not identified.

3.1.4 Rearrangement of Monoclonal T-Cell Receptor Gamma Locus

Clonality analyses were performed for 3 LS-associated SCCs, 7 LP-associated SCCs with adjacent d-PeIN, 2 HPV-positive PeINs with underlying LS and LP and 1 d-PeIN, which all contained T-lymphocyte dominant inflammatory infiltrates. The lymphatic infiltrate of the patient with LS and a HPV high-risk-39-, 68- and 72-positive PeIN revealed a single peak (172bp) indicating a monoclonal cell population after capillary electrophoresis with primers specific for T-gamma B (region Vg9+Jg1.3/2.3). No single peak was demonstrated in the other patient with HPV high-risk 16 (39)-positive PeIN and underlying LP. In 1 d-PeIN (Fig.20 c/d) and 3/7 LP-associated SCCs a prominent single peak indicating a mono-

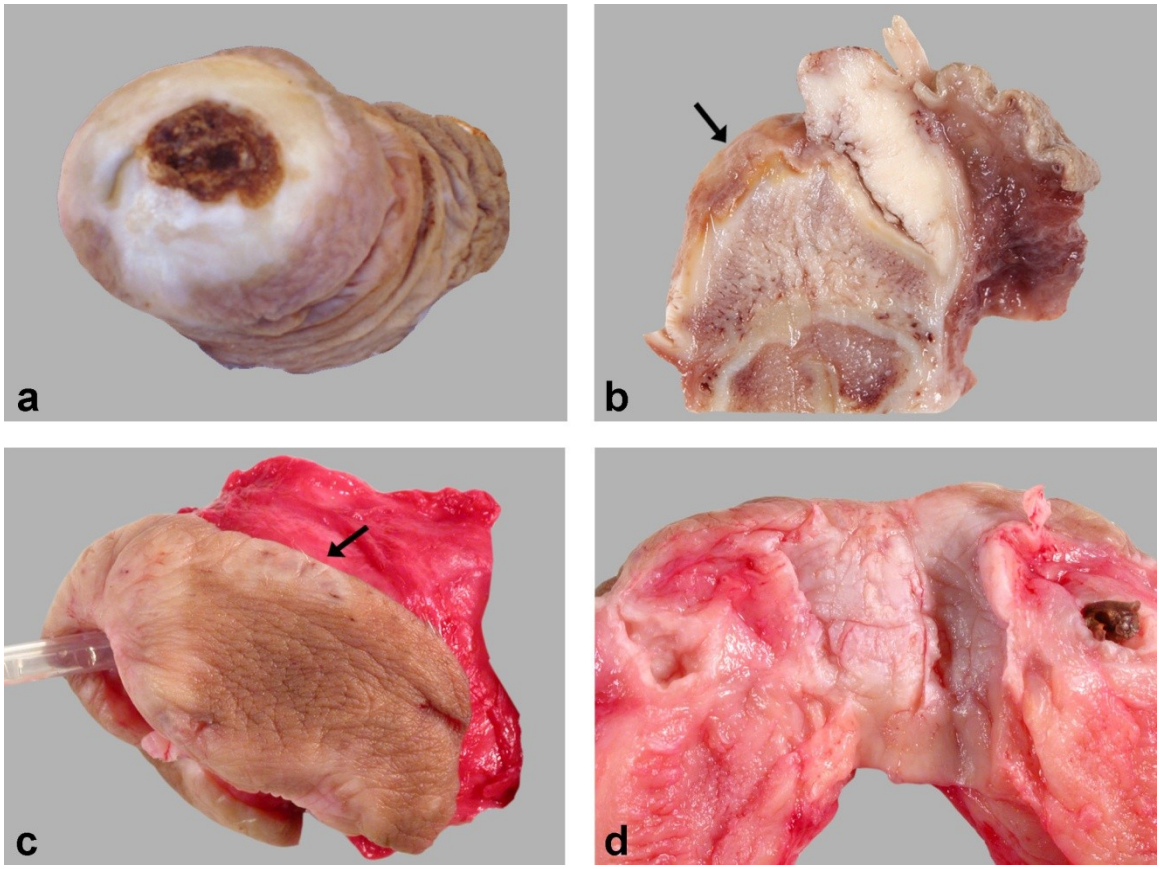
clonal cell population was identified with primers specific for T-gamma B (region Vh9+Jg1.3/2.3; 172bp). A prominent single peak indicating a monoclonal cell population with primers specific for T-gamma B (region Vh9+Jg1.3/2.3; 121bp) (Fig.19) was seen in 1/3 LS-associated SCCs. The inflammatory infiltrate of 1 LS-associated SCC and 1 LP-associated SCC revealed a single prominent peak suspicious of but not unequivocally diagnostic of monoclonality. Tissues of 1/3 LS-associated SCC and 1/7 LP-associated SCC did not reveal lymphocytes with monoclonally rearranged T-cell receptor gamma locus.

Figure 19: Clonality Analysis of Rearranged T-Cell Receptor Gamma Locus



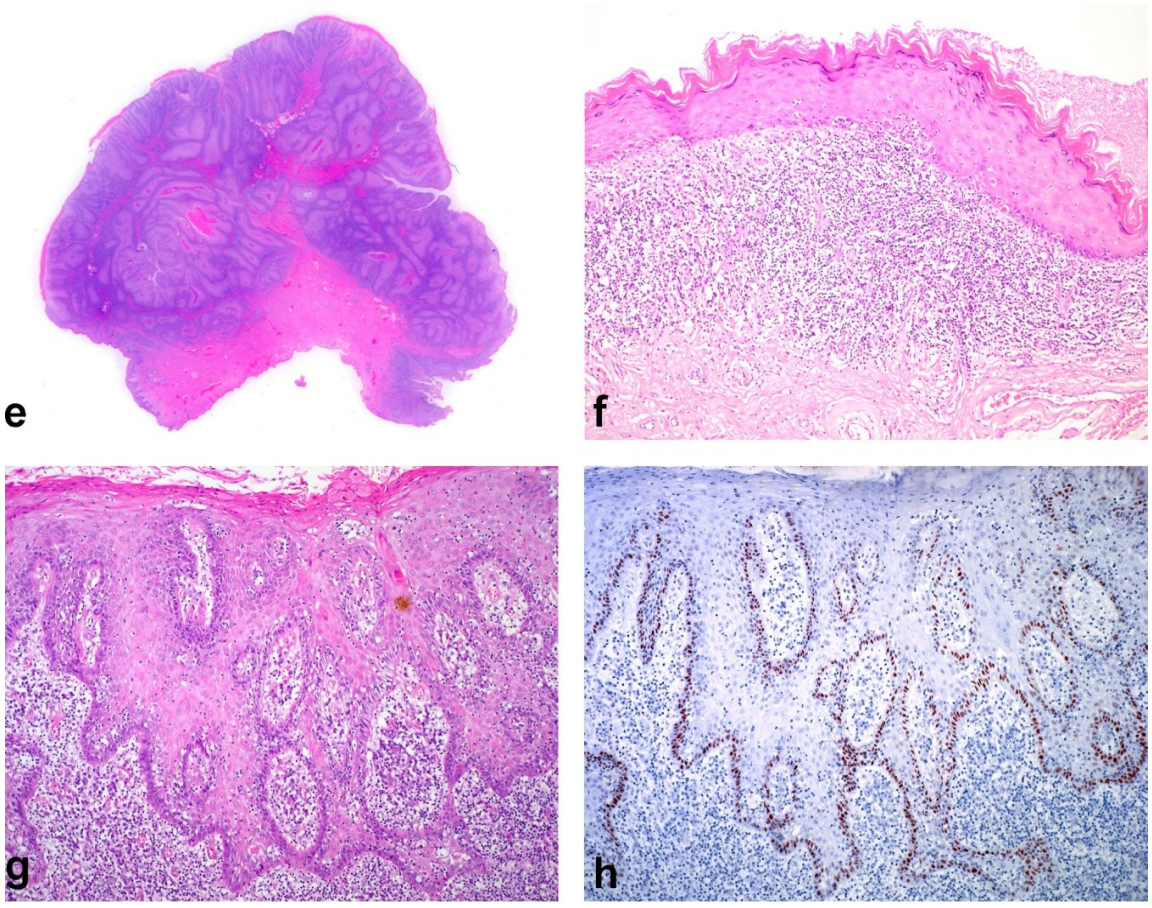
Clonality analysis from DNA isolated from paraffin embedded formalin fixed tumor tissue of a LS-associated pT2 SCC in a 80-year-old man with PCR using primers specific for T-cell receptor gamma locus. Conventional capillary electrophoresis revealed two prominent peaks at 54 base pairs und 59 base pairs (a). One prominent peak (121bp) was identified after capillary electrophoresis using Biomed-2 primers specific for T-gamma B (region Vg9+Jg1.3/2.3) (b).

Figure 20 A: HPV-negative differentiated Penile Intraepithelial Neoplasias and Squamous Cell Carcinomas



a) Distal penectomy specimen of a 67-year-old man with advanced LS and an ulcerated periurethral squamous cell carcinoma (pT1).
b) Formalin-fixed section of a HPV-negative penile SCC in a 47-year-man. The SCC arose in the inner side of the foreskin in the background of erosive LP of the glans penis. The formalin-fixed specimen revealed plaques with an orange brown centre and raised grey borders on the glans penis (arrow). In-vivo presentation would have been a "bright glazed erythema" with white lacy borders (Wickham's striae)
c) Penectomy specimen with flat confluent plaques of LP in the modified mucosa of the glans penis (arrow) and around meatus urethrae. Cyst formation and scarring are the results of longstanding LP. The patient had a circumcision for phimosis due to advanced hypertrophic LP 7 years earlier.
d) The cysts showed advanced hypertrophic LP with areas of d-PeIN. The distal urethra also shows confluent plaques and hyperkeratotic papules of longstanding LP.

Figure 20 B: HPV-negative differentiated Penile Intraepithelial Neoplasias and Squamous Cell Carcinomas



e) Polypoid SCC which arose at the sulcus coronarius in a 62-year-old man, who had been circumcised 3 years earlier for advanced LP with phimosis.

f) Atrophic LP adjacent to invasive SCC with flattened atrophic epithelium, hypergranulosis and hyperkeratosis, lichenoid infiltrate and basal keratinocyte apoptosis.

g) d-PeIN identified adjacent to LP-associated SCC of a 60-year-old man with elongated bridging rete ridges, premature squamatization, basal cell atypia and increased mitotic activity. The dense lymphohistiocytic infiltrate of invasive SCC and peritumoral d-PeIN contained lymphocytes with monoclonally rearranged T-cell receptor gamma locus.

h) p53 stains basal keratinocytes of d-PeIN

3.2 Prognostic Correlation of Histopathological Factors with Regional Lymph Node Metastases and Survival

3.2.1 Clinicopathological and Histopathological Parameters of 76 pT1 Penile Cancers (Summary Table 2)

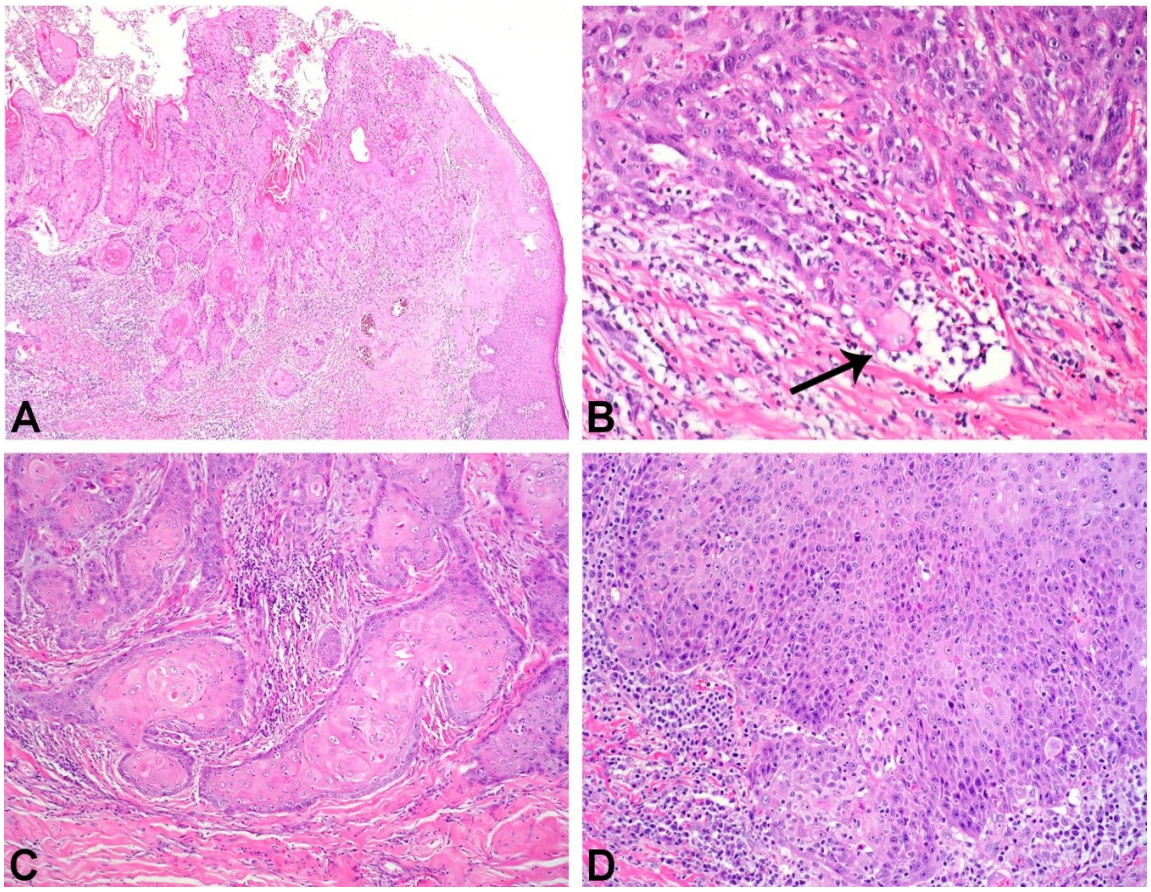
The 72 SCCs (95%; Fig.21A a-d) and 4 clear cell carcinomas (5%; Fig.21B e-f) were re-classified into 31pT1a SCCs (43%) and 45pT1b cancers (57%; 41 SCC and clear cell carcinomas). In the pT1a group, 10 SCCs were HPV-induced and 21/31 SCCs were HPV-negative SCCs mostly associated with dermatoses (Fig.21 a/b). In the pT1b group, all 4 clear cell carcinomas (Fig.21B e/f) and 36/41 SCCs (Fig.21A c/d) were HPV-induced, while only 5/45 pT1b cancers were HPV-negative. Of 22 patients, who underwent lymphadenectomy for enlarged lymph node, 12 men (55%) had metastases: 4/4 men with clear cell carcinomas (100%) and 8/72 men with SCC (11%; 2 pT1a, 6 pT1b). All clear cell carcinomas showed lymph vessel invasion (Fig.21B g) and lymph node metastases, which were of clear cell differentiation resembling the primary carcinoma. Perineural invasion was observed in all 4 clear cell carcinomas, but only in 7 SCC (Fig.20B h). Compared with conventional SCC within the pT1b group, clear cell carcinomas showed a significantly higher rate of lymphatic invasion (100% vs. 7%; Fig.20B g), perineural invasion (100% vs. 10%) and a higher degree of lymph node metastasis (100% vs. 15%). Despite the small number of clear cell carcinomas, all differences were statistically highly significant. No statistically significant difference was observed between pT1a and pT1b SCCs with respect to lymph vessel invasion, perineural invasion and lymph node metastasis. pT1b SCC showed a predominantly superficial growth pattern, while pT1a SCC showed a deeper invasion than pT1b.

Table 2: Histopathological Parameters and Lymph Node Status in pT1a and pT1b Penile Carcinomas

Parameter	SCC *(n=72)*			Clear cell carcinoma *(n=4)*	
	pT1a *(n=31)* n (% within pT1a)	pT1b *(n=41)* n (% within pT1b)	Significance pT1a vs. pT1b, p-value	pT1b *(n=4)* n (% within pT1b)	Significance vs. SCC pT1b, p-value
Grade:					
G1	16(53%)	0		0	
G2	14(47%)	1(2%)		0	
G3	0	38(91%)		4(100%)	
G4	0	3(7%)	**1.1E-14**	0	0.81
Dermatoses	15(56%)	8(19%)	**0.003**	4(100%)	1.0
HPV-DNA pos.	10(35%)	37(88%)	**5.9E-06**	4(100%)	1.0
Lymphatic invasion	0	3(7%)	0.26	4(100%)	**2.1E-04**
Lymph node metastasis	2(7%)	6(14%)	0.46	4(100%)	**0.001**
Perineural invasion	3(10%)	4(10%)	1.0	4(100%)	**4.3E-04**
Growth pattern:					
verrucous	6(20%)	3(7%)		0	
papillary	11(37%)	4(10%)		0	
superficial	13(43%)	35(83%)	**0.002**	4(100%)	0.68
Invasion depth:					
<=5 mm	11(37%)	28(67%)		0	
6-14 mm	10(33%)	11(26%)		4(100%)	
>=15 mm	9(30%)	3(7%)	**0.013**	0	**0.011**

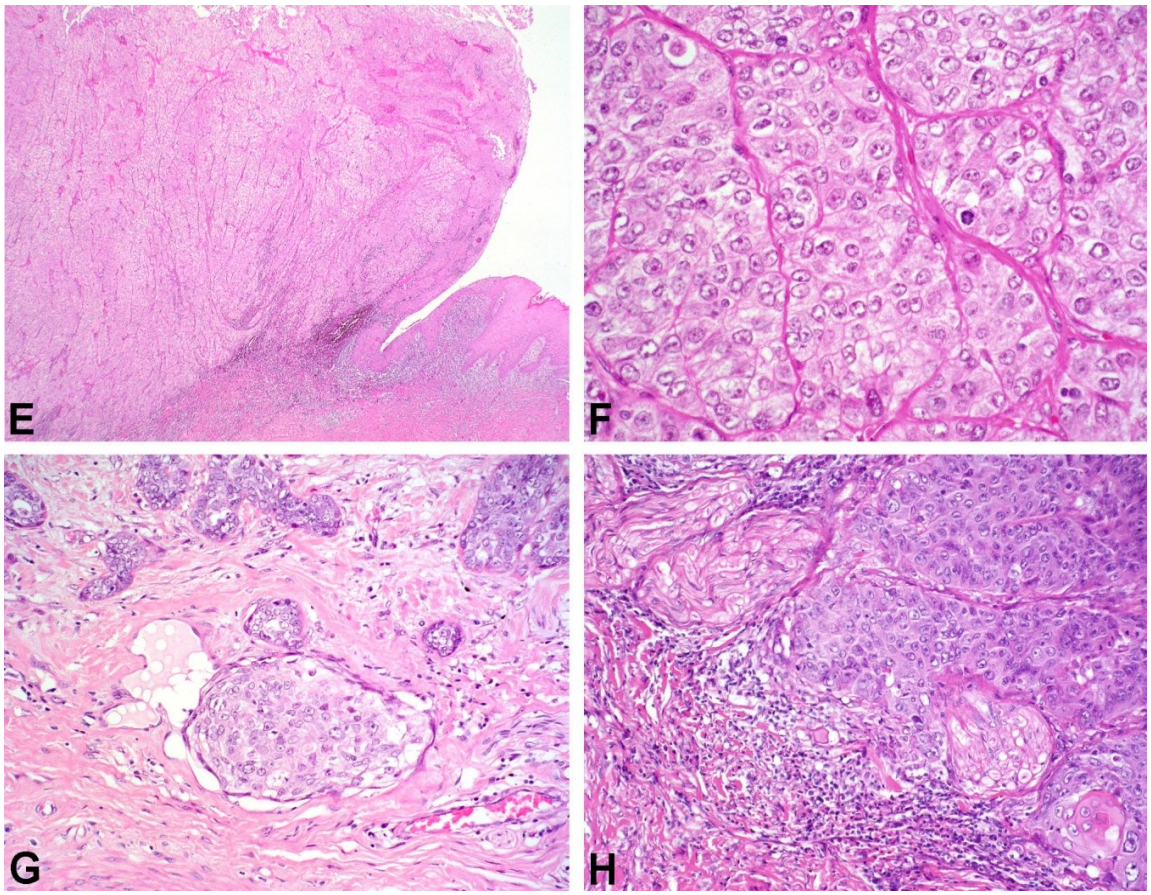
Statistically significant differences are written in **bold**.

Figure 21 A: HE Stains of pT1 Penile Cancers



a) Well-differentiated penile SCC, G1-2.
b) An individual large squamous tumor cell invades a lymphovascular space in a LS-associated SCC. The 67-yr-old patient had bilateral LN metastases.
c) HPV-negative penile SCC, G1-2 arising in the background of LS
d) HPV-negative SCC, G2 arising in LP without LN metastases in a 59-year-old man with a prior circumcision for highly active LP, 3 year earlier.

Figure 21 B: HE Stains of pT1 Penile Cancers



e) Low-power view of a clear cell penile carcinoma arising in the inner side of the foreskin of a 65-yr-old man, who had bilateral inguinal LN metastases. Note the sharp demarcation of the cancer to the surrounding squamous epithelium.
f) The carcinoma is highly vascularized and composed of clear cells proliferating in lobules.
g) Tumor embolus in lymph vessels of a HPV16-positive, predominant clear cell carcinoma in a 46-year-old man.
h) Extensive perineural invasion in a LP - associated SCC, G2-3 in a 48-year-old man, who underwent bilateral inguinal LN dissection without metastases during a follow-up of 47 months.

3.2.2 Clinicopathological Parameters of 72 SCC and Lymph Node Status (Summary Table 3)

Although clear cell cancer was statistically highly significantly associated with lymph node metastases, for sake of avoiding a biased analysis due to the rarity of these carcinomas, the analysis of predictive histological parameters was performed separately for conventional SCC. Detection of inguinal lymph node metastases in 8/18 SCCs after lymphadenectomy performed for enlarged lymph node corresponded to a 42% probability of correctly identifying metastasis. None of the patients with non-palpable lymph node (who therefore did not undergo lymph node dissection) developed lymph node metastases during the follow up of 47 months (range 5-265 months). Thus, clinical evaluation showed 100% sensitivity and a 100% negative predictive value for metastasis with only a 16% false-positive rate.

The only histological parameter significantly associated with lymph node metastases was lymphatic invasion in the primary SCC (Fig. 22 b; 100% specificity and positive predictive value). The sensitivity of lymphatic invasion was low, as in 5/8 metastasized SCCs (63%) no lymph vessel invasion on HE stained section and immunohistochemical analysis with antibody to D2-40 was detected. No significant association was observed between lymph node metastases and the remaining tested parameters: pT1 subcategories, tumor grade, perineural invasion, growth pattern, invasion depth, HPV-status and presence of dermatoses. Tumor grades were evaluated alone and in various combinations (single grades or two tiered groups G1 versus G2-4 and G1/2 versus G3/4), but were not statistically significantly associated with lymph node metastases. Lymphatic invasion occurred independent of tumor grades, which is exemplified by an individual large keratinized tumor cells of a LS-associated SCC, G2 invading a lymphatic space (Fig.21A b).

Table 3: Predictive Value of Clinicopathological Parameters for Lymph Node Metastasis in pT1a and pT1b Penile Squamous Cell Carcinomas (clear cell carcinomas are excluded)

Predictor of pN1	**pN0 (n=64)**	**pN1 (n=8)**	**Significance, p-value**	**PPV***	**NPV***
Clinically enlarged lymph nodes	10(16%)	8(100%)	**3.7E-06**	44% (8/18)	100% (54/54)
Lymphatic invasion	0	3(38%)	**0.001**	100% (3/3)	93% (64/69)
pT1b	36(56%)	6(75%)	0.46		
G1 versus G2/3/4	49(77%)	7(88%)	0.67		
G1/2 versus G3/4	36(56%)	5(63%)	1.0		
Perineural invasion	5(8%)	2(25%)	0.17		
Growth pattern:					
verrucous	6(9%)	3(38%)			
papillary	13(20%)	2(25%)			
superficial	45(70%)	3(38%)	0.06		
Invasion depth:					
<=5 mm	36(56%)	3(38%)			
6-14 mm	18(28%)	3(38%)			
>=15 mm	10(16%)	2(25%)	0.59		
HPV-DNA pos.	43(68%)	4(50%)	0.43		
Dermatoses	19(31%)	4(50%)	0.43		

*PPV (Positive predictive value) and NPV (Negative predictive value) are given only for those parameters that have a statistically significant association with lymph node status. Statistically significant associations are in **bold**.

3.2.3 Survival Analysis of 76 pT1 Penile Cancers

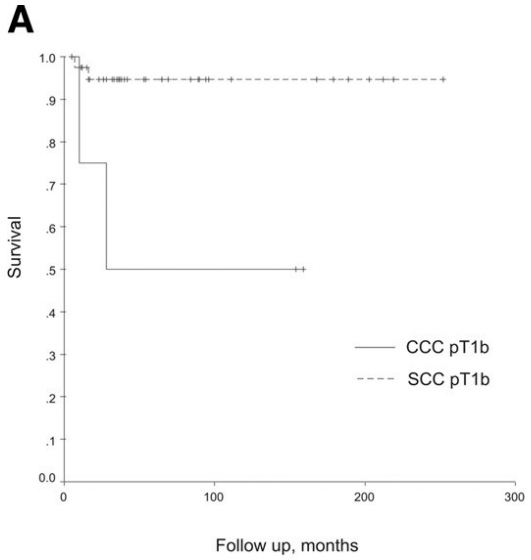
Median follow-up was 47 months (range 5-265 months) during which 5/76 patients died of disease (6.6%; 3 SCC, 2 clear cell carcinomas). Of the 4 men with clear cell carcinoma, one is alive after 10 years and one died of liver cirrhosis after 4 years. One man with clear cell carcinoma and bilateral conglomerate metastases died of metastatic disease to liver and lung. The other patient with clear cell carcinoma and 1 with SCC died of uncontrollable extensive local and abdominal disease with direct infiltration of inguinal tissues, pelvic muscles, bone and metastases to aortic and retroperitoneal lymph node. Two men with SCC died of massive tumor hemorrhage after infiltration of blood vessels in the inguinal basin. Overall, patients with clear cell carcinomas revealed a poorer survival than patients with pT1b SCC (p=0.004, Fig. 22 a). Univariate survival analysis for SCC alone showed a significant association between survival and presence of lymph node metastasis (p<0.00005), lymph vessel invasion (p=0.0007) and growth pattern (p=0.01), Fig. 22 b-c). No association was observed between survival and pT1-subcategory, tumor grade alone (in any variation), perineural invasion, invasion depth and HPV status.

Table 4: Association of Clinicopathological Parameters with Survival in pT1a and pT1b Penile Squamous Cell Carcinomas

Parameter	Log rank test, p-value
LN metastasis	**<0.00005**
Lymph vessel invasion	**0.0007**
Growth pattern	**0.01**
pT1a vs. pT1b	0.64
G1 vs. G2/3/4	0.91
G1/2 vs. G3/4	0.51
Perineural invasion	0.12
Invasion depth	0.38
HPV-DNA-pos.	0.26
Dermatoses	0.83

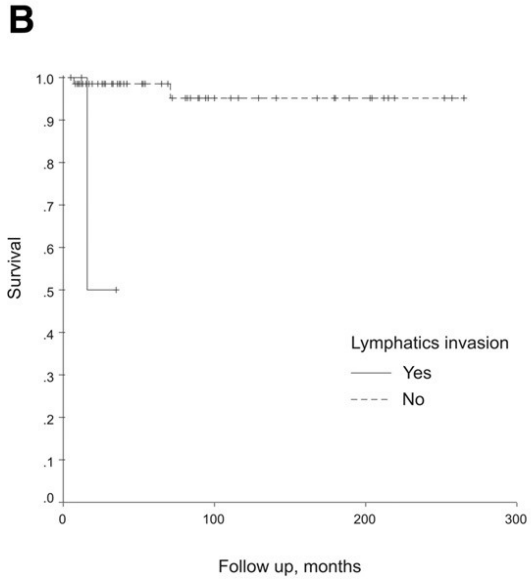
Statistically significant associations are in **bold**.

Figure 22: Survival Analysis of 76 pT1 Penile Cancers

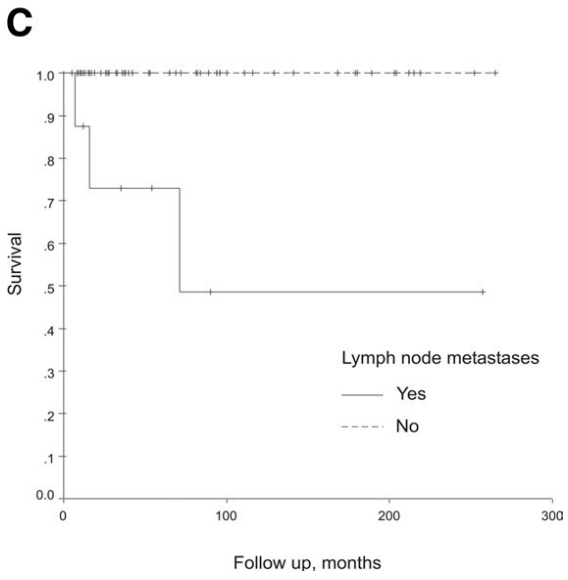


Kaplan-Meier survival analysis of pT1 penile carcinomas: Comparison of clear cell carcinomas (CCC) versus SCC within the same pT-category:

(A) Survival of pT1b clear cell carcinomas versus pT1b squamous cell carcinoma shows a statistically significant difference ($p = 0.004$)



Analysis of penile squamous cell carcinomas excluding clear cell carcinomas:
(B) Lymph vessel invasion in penile squamous cell carcinomas had a statistically significant impact on survival ($p=0.0007$)



(C) Presence of lymph node metastasis in penile squamous cell carcinomas had a statistically significant impact on survival ($p<0.00005$)

4 Discussion

4.1 Dermatoses Are Under-appreciated in Penile Carcinogenesis

We identified 2 major pathways in penile carcinogenesis in a low incidence area of penile cancer: HPV and dermatoses. 60% of invasive penile cancers were HPV-induced, slightly higher than the reported global HPV prevalence of 46.9% [8]. Over 80% of our patients had single genotype infections with 80% accounting for HPV high-risk 16, 6% for HPV high-risk 33, 4% for HPV high-risk 18 and 2% for HPV high-risk 45. Compared to the reported global relative contributions of HPV 16 (60%), HPV 18 (13%), HPV 6/11 (8%), HPV 31 (1%), HPV 45 (1%), HPV 33 (1%), HPV 52 (0.6%) [8], we observed a higher percentage of HPV high-risk 16 and HPV high-risk 33, but a lower percentage of HPV high-risk 18. With the exception of one HPV high-risk-45-positive but repeatedly p16 ^{INK4a} -negative SCC, p16^{INK4a} over-expression correlated with detection of HPV high-risk genotypes, implicating HPV as the etiologic agent in these cancers. Presently available vaccinations against HPV high-risk 16/18 could have prevented about 50% of all penile cancers in our patient cohort.

LS and in particular LP are underappreciated as possible risk factors for penile carcinoma. Forty percent of invasive SCCs were HPV-negative and associated with advanced LS and LP. LS was more common, but LP was the underlying dermatoses in 20% of HPV-negative SCC. While LS has been reported as risk factor for penile SCC, there are few publication about penile LP as a precancerous lesion [49]. In mucosal LP the risk for malignant transformation is up to 5% [50,51]. Penile skin and keratinized mucosa of glans penis are often involved by annular papules or confluent plaques of LP. In contrast to LS, LP often involves mucosal surfaces including the meatus urethrae and urethra. Mucosal LP often presents in the "erosive" form with constant regeneration and wound healing leading to scarring and urethral strictures. The majority of penile cancers arising in LS and LP had dense lymphocytic infiltrates including a lymphocytic vasculitis [23,30]. We were able to document for the first time the presence of T-cells with monoclonally rearranged T-cell receptor gamma locus in penile LS- and LP-associated SCCs. Accumulations of T-lymphocytes with monoclonally rearranged T-cell receptor gamma locus have been described for LP [29,52], LS and LS -associated vulvar SCC [29,52], sometimes reaching up to 20% of the entire T-cell infiltrate [53]. It remains unclear what or which antigen drives the immune system. We

speculate that the immune deregulation contributes to the carcinogenesis of anogenital SCC arising in LS and LP via reduced T-cell diversity within tumor infiltrating lymphocytes which creates a permissive environment for rapid progression from d-PeIN to invasive SCC.

Precursor lesions of HPV-negative anogenital SCC are poorly defined, but atypical well-differentiated squamous intraepithelial lesions called differentiated VIN and d-PeIN are believed to be precursors with a rapid progression to invasive SCC, often in less than 6 months [33,54]. This may explain the high percentage of pT2 and pT3 carcinomas among HPV-negative cancers and the low detection rate of isolated d- PeIN without SCC. Men with penile LS and LP should be closely monitored for development of suspicious lesion. For this reason, we have established a *"Penile dermatosis clinic"* at the Department of Urology, Medical University Graz, where men with a diagnosis of LS and LP after circumcision are thoroughly examined for residual clinical lesions and regularly followed. Forty percent of HPV-negative penile SCC arising in advanced dermatoses represents a huge burden of possibly preventable cancers, if early disease stages are recognized and appropriately treated [25,55,56].

It was interesting, that 10% of PeIN and 1 micro-invasive SCC were incidental findings of only few millimeters in foreskins after circumcision for phimosis in young patients. They were not visible clinically and in formalin-fixed specimens but detected only after histological examination of the entire specimen. These small lesions will go undetected if the foreskin is not submitted to histological examination, which is still common practice in some hospitals and practices. They will also go undiagnosed when only a random section is submitted for histological examination, which is standard procedure in many pathology laboratories.

4.2 Penile Clear Cell Carcinomas Are more Aggressive than Squamous Cell Carcinomas

In agreement with other studies, only 55% of patients with pT1 penile cancer undergoing inguinal lymphadenectomy had lymph node metastases. These included 100% of patients with clear cell carcinomas, but only 11% with SCC. All men with clear cell carcinomas demonstrated lymphatic invasion, perineural invasion and lymph node metastases along with a disease-related mortality of 50%. Penile clear cell carcinomas are similarly aggres-

sive cancers as clear cell carcinomas in other organs such as distal urethra [57] and skin [58] and are more aggressive than penile SCC. Penile clear cell carcinomas have been observed as single cases in the past [59], but have recently become recognized as an entity in urologic and pathologic text books, such as the 2011 Armed Forces Institute of Pathology Atlas on tumors of lower male genital tract [43]. The only series of clear cell carcinomas to date has been described in patients from Styria [9,60]. An explanation for the relatively large number of penile clear cell carcinoma in this area may come from the unique medical situation in the state of Styria during the past 25 years. The Institute of Pathology at the University of Graz, one of the largest in Europe, has served the entire state of Styria with about 1 million residents until several years ago as there were no private practices or other major hospital pathologies. The evaluated penile cancers in this study and in previous publications from Styria [9,60] therefore represent a true cross section through a large homogenous population. This fact may explain the discrepancy between the relatively large number of clear cell carcinomas in our patient collective and the observation of single cases only at other institutions serving much smaller communities. Other reasons may be that clear cell carcinomas were not reported as such during routine sign out (which happened initially with 2 of our cases), that they were not recognized due to insufficient tumor sampling or that they were diagnosed, but not published. Despite the small numbers, our observations suggest that clear cell differentiation *per se* in penile cancers justifies an inguinal lymphadenectomy. A multi-institutional review effort of this rare entity may be helpful to collect more cases and to obtain a better understanding of penile clear cell carcinomas. For the time being, generalization from this small cancer group should be cautious.

4.3 Only Lymphovascular Invasion but not the TNM Classification in the Primary Cancer Predicted Presence of Lymph Node Metastases with 100% Specificity

The revised 2009 TNM classification was not as accurate in predicting lymph node status as clear cell differentiation and clinical investigation of the groins. Among the tested histological parameters, only lymphovascular invasion in the primary cancer predicted presence of lymph node metastases with 100% specificity, but poor sensitivity. Reliable detection of lymph vessel invasion depends on the extent of routine surgical work-up, but step sectioning and immunohistochemical analysis additionally performed for this study did not improve detection rate. For conventional SCC, neither perineural invasion nor depth of invasion or growth pattern were associated with lymph node status, similar to findings of

Cubilla [3]. We could not confirm observations of Velazquez and colleagues [44] that perineural invasion alone was associated with survival or presence of lymph node metastases. Tumor grade alone of the primary SCC, independent of two-, three- and four-tiered grading schemes, could not identify patients with metastases, but correlated well with etiology, e.g. dermatoses-associated HPV-negative SCC were found predominantly in pT1a and HPV-induced SCC in pT1b. In analogy to G1 and G2 vulvar LS-associated SCC, where keratinized tumor cells with diameters between 30 and 60µm are capable of lymph vessel invasion, implantation and proliferation in lymph nodes [61,62], single keratinized tumor cells of dermatoses-associated HPV-negative penile SCC can invade lymph spaces. Our observations suggest that after a careful histopathological work-up of the entire primary SCC for exclusion of lymphatic invasion, a clinically node-negative patient may be followed expectantly independent of the tumor grade of the penile SCC.

The reasons for the low probability (42%) of metastases in patients with SCC and enlarged lymph node in the absence of infections remain largely unclear. For HPV-negative SCC, the underlying dermatoses may offer an explanation. LS and LP are immune dysregulations with accumulation of lymphocytes sharing a monoclonally rearranged T-cell receptor [23,29,52,53,60,63] as the result of an exuberant local immune reaction. The constant trafficking of lymphocytes between anogenital skin and regional lymph node may lead to clinically enlarged regional lymph node. On the other hand, the reduced T-cell diversity in dermatoses and differentiated penile intraepithelial precursor lesions may lead to early immunological escape of individual invasive malignant tumor cells. Although none of our patients with clinically node-negative inguinal basins developed lymph node metastases (independent of subclassification in pT1a or pT1b) this may be an explanation for "occult metastases" in patients who were followed expectantly based on low tumor grade [37]. These patients would be perfect candidates for dynamic sentinel lymph node identification [64-67].

4.4 Summary Conclusions

In summary, we identified 2 major pathways for penile cancer in 115 patients in Styria, a low incidence area for penile carcinoma. A transforming HPV-infection accounted for 60% of invasive penile cancers, with a predominance of HPV high-risk 16 as single genotype in 80% of all HPV-induced penile cancers suggesting a beneficial effect of the presently available HPV-vaccination. Forty percent of penile SCCs were HPV-negative. Most arose in the background of advanced LS and LP, were overrepresented in advanced pT2 and pT3 tumor stages, associated with d-PeIN and accumulations of T-lymphocytes with

monoclonally rearranged T-cell receptor gamma locus. LS and particularly LP are under-recognized as possible cancer risk and represent a huge burden of possibly preventable cancers, if early disease stages are appropriately treated and patients regularly followed. Penile clear cell carcinomas are more aggressive than SCC. Clear cell differentiation in primary penile cancer was prognostic of lymph node metastases in 100% and associated with poor survival in 50%. For conventional penile SCC, only lymphatic invasion correlated with presence of lymph node metastases, but not sub-classification according to 2009 TNM, tumor grade alone, perineural invasion, growth pattern and invasion depth. Despite poor sensitivity, clinical evaluation of the inguinal basin remains the superior staging method in penile cancer when compared to risk stratification based on histological parameters. The revised 2009 TNM classification is inaccurate for predicting lymph node metastases in early stage cancers.

5 References

1 Hernandez BY, Barnholtz-Sloan J, German RR *et al.* Burden of invasive squamous cell carcinoma of the penis in the United States, 1998-2003. *Cancer* 2008; **113**: 2883-91.

2 Madsen BS, van den Brule AJ, Jensen HL *et al.* Risk factors for squamous cell carcinoma of the penis--population-based case-control study in Denmark. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 2683-91.

3 Cubilla AL. The role of pathologic prognostic factors in squamous cell carcinoma of the penis. *World J Urol* 2009; **27**: 169-77.

4 Eble JE, Sauter G, Epstein JI *et al.* Tumours of the Urinary System and Male Genital Organs In: *World Health Organisation of Tumours. Pathology and Genetics* (Organisation WH, ed). Lyon: IARC Press. 2004; 279-90.

5 Epstein JI, Cubilla AL, Humphrey PA, eds. *Tumors of the prostate gland, seminal vesicles, penis, and scrotum.*, Vol. 14. Washington, D.C: Armed Forces Institute of Pathology. 2011.

6 Chaux A, Pfannl R, Lloveras B *et al.* Distinctive association of p16INK4a overexpression with penile intraepithelial neoplasia depicting warty and/or basaloid features: a study of 141 cases evaluating a new nomenclature. *Am J Surg Pathol* 2010; **34**: 385-92.

7 Chaux A, Reuter V, Lezcano C *et al.* Comparison of morphologic features and outcome of resected recurrent and nonrecurrent squamous cell carcinoma of the penis: a study of 81 cases. *Am J Surg Pathol* 2009; **33**: 1299-306.

8 Miralles-Guri C, Bruni L, Cubilla AL *et al.* Human papillomavirus prevalence and type distribution in penile carcinoma. *J Clin Pathol* 2009; **62**: 870-8.

9 Liegl B, Regauer S. Penile clear cell carcinoma: a report of 5 cases of a distinct entity. *Am J Surg Pathol* 2004; **28**: 1513-7.

10 Rubin MA, Kleter B, Zhou M *et al.* Detection and typing of human papillomavirus DNA in penile carcinoma: evidence for multiple independent pathways of penile carcinogenesis. *Am J Pathol* 2001; **159**: 1211-8.

11 Backes DM, Kurman RJ, Pimenta JM *et al.* Systematic review of human papillomavirus prevalence in invasive penile cancer. *Cancer Causes Control* 2009; **20**: 449-57.

12 Rose RC, Stoler MH. Biologie. In: *Leitfaden zu Genitale HPV-Erkrankungen und deren Prävention* (Bonnez W, ed). New York: Informa healthcare. 2009; 1-16.

13 Bonnez W, Toy EP. Krankheiten. In: *Leitfaden zu Genitale HPV-Erkrankungen und deren Prävention* (Bonnez W, ed). New York: informa helthcare. 2009; 31-48.

14 Burchell AN, Franco EL. Epidemiology of oncogenic and nononcogenic HPV types, and the evidence for differences in their sexual behavior. In: *Emerging issues in HPV infections* (Monsenego J, ed). Paris: Karger. 2006; 20-33.

15 Don N, ed. *Human Pipllomavirus (HPV)*. USA: Thomson Gale. 2007.

16 society AC. What are the risk factors for cervical cancer? In. 2012.

17 Reuschenbach M, Seiz M, Doeberitz CK *et al.* Evaluation of cervical cone biopsies for coexpression of p16(INK4a) and Ki-67 in epithelial cells. *Int J Cancer* 2012; **130**: 388-94.

18 Wells M, Östör AG, Crum AP *et al.* Epithelial Tumours. In: *Pathology and Genetics:Tumours of the breast and female genital organs* (Tavassoli FA, Devilee P, eds), Vol. 5. Lyon: IARC Press. 2003; 262-79.

19 Trunk MJ, Dallenbach-Hellweg G, von Knoebel Doeberitz M. CINtec^{TM} p16^{INK4a} Staining Atlas. In.

20 Bunker CB. Diseases and disorders of the male genitalia. In: *Fitzpatrick's Dermatology in General Medicine* (Wolff K, Goldsmith LA, Katz SI et al., eds), 7 edn., Vol. 1: McGrawHill Medical. 2008; 654-75.
21 Powell JJ, Wojnarowska F. Lichen sclerosus. *Lancet* 1999; **353**: 1777-83.
22 Becker K. Lichen sclerosus in boys. *Dtsch Arztebl Int*; **108**: 53-8.
23 Regauer S. Immune dysregulation in lichen sclerosus. *Eur J Cell Biol* 2005; **84**: 273-7.
24 Stühmer A. Balanitis xerotica obliterans (postoperationem) und ihre Beziehung zur "Kraurosis glandis et preputii penis". *Arch Derm Syph (Berlin)* 1928; **156**: 613-23.
25 Neill SM, Tatnall FM, Cox NH. Guidelines for the management of lichen sclerosus. *Br J Dermatol* 2002; **147**: 640-9.
26 Regauer S, Liegl B, Reich O. Author's Reply: Elastic fibers in lichen sclerosus. *Histopathology* 2007; **50**: 388-9; author reply 9-91. .
27 Mercado R, Vijh S, Allen SE *et al.* Early programming of T cell populations responding to bacterial infection. *The Journal of Immunology* 2000; **165**: 6833-9.
28 Baker BS, Brown DW, Fischetti VA *et al.* Skin T cell proliferative response to M protein and other cell wall and membrane proteins of group A streptococci in chronic plaque psoriasis. *Clin Exp Immunol* 2001; **124**: 516-21.
29 Regauer S, Reich O, Beham-Schmid C. Monoclonal gamma T-cell receptor rearrangement in vulvar lichen sclerosus and squamous cell carcinomas. *Am J Pathol* 2002; **160**: 1035-45.
30 Regauer S, Liegl B, Reich O *et al.* Vasculitis in lichen sclerosus: an under recognized feature? *Histopathology* 2004; **45**: 237-44.
31 Pittelkow MR, Daoud MS. Lichen Planus. In: *Fitzpatrick's Dermatology in Gender Medicine* (Wolff K, Lowell A, Goldsmith LA et al., eds). 2008; 244-55.
32 Weedon D, ed. *Weedon's Skin Pathology*, Vol. 2: Churchill Livingstone Elsevier. 2010.
33 Velazquez EF, Cubilla AL. Lichen sclerosus in 68 patients with squamous cell carcinoma of the penis: frequent atypias and correlation with special carcinoma variants suggests a precancerous role. *Am J Surg Pathol* 2003; **27**: 1448-53.
34 Regauer S. Residual anogenital lichen sclerosus after cancer surgery has a high risk for recurrence: a clinicopathological study of 75 women. *Gynecol Oncol*; **123**: 289-94.
35 Coblentz TR, Theodorescu D. Morbidity of modified prophylactic inguinal lymphadenectomy for squamous cell carcinoma of the penis. *J Urol* 2002; **168**: 1386-9.
36 Deem S, Keane T, Bhavsar R *et al.* Contemporary diagnosis and management of squamous cell carcinoma (SCC) of the penis. *BJU Int* 2011; **108**: 1378-92.
37 Malhotra SM, Rouse RV, Azzi R *et al.* Is lymphadenectomy indicated in patients with T1 moderately differentiated penile cancer? *Can J Urol* 2009; **16**: 4895-900.
38 Pizzocaro G, Algaba F, Horenblas S *et al.* EAU Penile Cancer Guidelines 2009. *Eur Urol* 2010; **57**: 1002-12.
39 Rossari JR, Vora T, Gil T. Advances in penile cancer management. *Curr Opin Oncol* 2010; **22**: 226-35.
40 Sobin L, Gospodarowicz M, Wittekind C, eds. *TNM Calssification of Malignant Tumors*, 7 edn.: Wiley-Blackwell. 2010.
41 Hughes BE, Leijte JA, Kroon BK *et al.* Lymph node metastasis in intermediate-risk penile squamous cell cancer: a two-centre experience. *Eur Urol* 2009; **57**: 688-92.
42 Chaux A, Soares F, Rodriguez I *et al.* Papillary squamous cell carcinoma, not otherwise specified (NOS) of the penis: clinicopathologic features, differential diagnosis, and outcome of 35 cases. *Am J Surg Pathol* 2010; **34**: 223-30.

43 Epstein JI, Cubilla AL, Humpfrey PA, eds. *Tumors of the penis*, Vol. Fascicle 14. Washington D.C., USA: American Registry of Pathology in collaboration with the Armed Forces Institute of Pathology. 2011.
44 Velazquez EF, Ayala G, Liu H *et al.* Histologic grade and perineural invasion are more important than tumor thickness as predictor of nodal metastasis in penile squamous cell carcinoma invading 5 to 10 mm. *Am J Surg Pathol* 2008; **32**: 974-9.
45 Chaux A, Caballero C, Soares F *et al.* The prognostic index: a useful pathologic guide for prediction of nodal metastases and survival in penile squamous cell carcinoma. *Am J Surg Pathol* 2009; **33**: 1049-57.
46 Sobin LH, Gospodarowicz MK, Wittekind C, eds. *TNM classification of malignant tumours*, 7th Edition: Wiley-Blackwell. 2009.
47 Munoz N, Bosch FX, de Sanjose S *et al.* Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; **348**: 518-27.
48 Salisbury CL, Budnick SD, Li S. T-cell receptor gene rearrangement and CD30 immunoreactivity in traumatic ulcerative granuloma with stromal eosinophilia of the oral cavity. *Am J Clin Pathol* 2009; **132**: 722-7.
49 Fundaro S, Spallanzani A, Ricchi E *et al.* Squamous-cell carcinoma developing within anal lichen planus: report of a case. *Dis Colon Rectum* 1998; **41**: 111-4.
50 Gandolfo S, Richiardi L, Carrozzo M *et al.* Risk of oral squamous cell carcinoma in 402 patients with oral lichen planus: a follow-up study in an Italian population. *Oral Oncol* 2004; **40**: 77-83.
51 Chryssostalis A, Gaudric M, Terris B *et al.* Esophageal lichen planus: a series of eight cases including a patient with esophageal verrucous carcinoma. A case series. *Endoscopy* 2008; **40**: 764-8.
52 Shiohara T, Mizukawa Y, Takahashi R *et al.* Pathomechanisms of lichen planus autoimmunity elicited by cross-reactive T cells. *Curr Dir Autoimmun* 2008; **10**: 206-26.
53 Regauer S, Beham-Schmid C. Detailed analysis of the T-cell lymphocytic infiltrate in penile lichen sclerosus: an immunohistochemical and molecular investigation. *Histopathology* 2006; **48**: 730-5.
54 Renaud-Vilmer C, Cavelier-Balloy B, Verola O *et al.* Analysis of alterations adjacent to invasive squamous cell carcinoma of the penis and their relationship with associated carcinoma. *J Am Acad Dermatol* 2010; **62**: 284-90.
55 Moyal-Barracco M, Edwards L. Diagnosis and therapy of anogenital lichen planus. *Dermatol Ther* 2004; **17**: 38-46.
56 Pugliese JM, Morey AF, Peterson AC. Lichen sclerosus: review of the literature and current recommendations for management. *J Urol* 2007; **178**: 2268-76.
57 Oliva E, Young RH. Clear cell adenocarcinoma of the urethra: a clinicopathologic analysis of 19 cases. *Mod Pathol* 1996; **9**: 513-20.
58 Wong TY, Suster S, Nogita T *et al.* Clear cell eccrine carcinomas of the skin. A clinicopathologic study of nine patients. *Cancer* 1994; **73**: 1631-43.
59 Young RH, Srigley LR, Amin MB, eds. *Tumors of the prostate gland, seminal vesicles, male urethra, and penis.* . Washington, D. C. USA,: AFIP. 2000.
60 Mannweiler S, Sygulla S, Beham-Schmid C *et al.* Penile carcinogenesis in a low incidence area: A clinicopathological and molecular analysis of 115 invasive carcinomas with special emphasis on chronic inflammatory skin diseases. *Am J Surg Pathol* 2011; **35**: 998-1006.
61 Tjin Asjoe FM, van Bekkum E, Ewing P *et al.* Sentinel node procedure in vulvar squamous cell carcinoma: a histomorphologic review of 32 cases. The significance

of anucleate structures on immunohistochemistry. *Int J Gynecol Cancer* 2008; **18**: 1032-6.

62 Regauer S. Histopathological work-up and interpretation of sentinel lymph nodes removed for vulvar squamous cell carcinoma. *Histopathology* 2009; **55**: 174-81.

63 Lukowsky A, Muche JM, Sterry W *et al.* Detection of expanded T cell clones in skin biopsy samples of patients with lichen sclerosus et atrophicus by T cell receptor-gamma polymerase chain reaction assays. *J Invest Dermatol* 2000; **115**: 254-9.

64 Hadway P, Smith Y, Corbishley C *et al.* Evaluation of dynamic lymphoscintigraphy and sentinel lymph-node biopsy for detecting occult metastases in patients with penile squamous cell carcinoma. *BJU Int* 2007; **100**: 561-5.

65 Graafland NM, Lam W, Leijte JA *et al.* Prognostic Factors for Occult Inguinal Lymph Node Involvement in Penile Carcinoma and Assessment of the High-Risk EAU Subgroup: A Two-Institution Analysis of 342 Clinically Node-Negative Patients. *Eur Urol* 2010; **58**: 742-7

66 Leijte JA, Hughes B, Graafland NM *et al.* Two-center evaluation of dynamic sentinel node biopsy for squamous cell carcinoma of the penis. *J Clin Oncol* 2009; **27**: 3325-9.

67 Graafland NM, Verhoeven RH, Coebergh JW *et al.* Incidence trends and survival of penile squamous cell carcinoma in the Netherlands. *Int J Cancer* 2010; **128**: 426-32.

6 Publications

Search word: Sygulla, 2009. Hits: 14

2012

Mannweiler, S; Sygulla, S; Tsybrovskyy, O; Razmara, Y; Pummer, K; Regauer, S Clear-Cell differentiation and lymphatic invasion, but not the revised TNM classification, predict lymph node metastases in pT1 penile cancer: A clinicopathologic study of 76 patients from a low incidence area.
Urol Oncol. 2012; 68(Pt 3): PubMed FullText FullText_MUG

The first and second author contributed equally to this paper

2011

Manneiler, S; Sygulla, S; Razmara, Y; Pummer, K; Regauer, S Differenzierte penile intraepitheliale Neoplasie (d-PeIN) als Vorläuferläsionen für Dermatosen-assoziierte Peniskarzinome.
Der Urologe. 2011; Suppl. 1(50):-63. Kongress der Deutschen Gesellschaft für Urologie; Sept 14 - 17, 2011; Hamburg, Germany. [Poster]

Mannweiler, S; Sygulla, S; Beham-Schmid, C; Razmara, Y; Pummer, K; Regauer, S Penile Carcinogenesis in a Low-Incidence Area: A Clinicopathologic and Molecular Analysis of 115 Invasive Carcinomas With Special Emphasis on Chronic Inflammatory Skin Diseases
AMER J SURG PATHOL. 2011; 35(7): 998-1006. PubMed FullText FullText_MUG

Mannweiler, S; Sygulla, S; Razamara, Y; Pummer, K; Regauer, S Penile Karzinogese in einem "low incidence" Gebiet für penile Karzinome: Eine transformierende HPV-high-risk Infektionen ist für 60% der steirischen Peniskarzinome vernantwortlich.
Der Urologe. 2011; Suppl. 1(50):-63. Kongress der Deutschen Gesellschaft für Urologie; Sept 14 - 17, 2011; Hamburg, Germany. [Poster]

Mannweiler S, Sygulla S, Winter E, Razmara K, Pummer K, Regauer S Penile Karzinogese: Transformierende HPV-High-Risk-Infektionen und Dermatosen (LS und LP)
Journal für Urologie und Urogynäkologie. 2011; 18, Sonderheft 1: 27-27.-37. Gemeinsame Tagung der Österreichischen Gesellschaft für Urologie und Andrologie und der Bayerischen Urologenvereinigung; JUN 2 - 4, 2011; Klagenfurt, AUSTRIA. [Oral Communication]

Mannweiler, S; Sygulla, S; Winter, E; Razmara, Y; Pummer, K; Regauer, S Distribution of Human Papilloma Virus Genotypes and Dermatoses in Penile Carcinoma in a Low Incidence Area in Europe
MODERN PATHOL. 2011; 24: 884 [Poster]

Mannweiler, S; Sygulla, S; Winter, E; Razmara, Y; Pummer, K; Regauer, S Distribution of Human Papilloma Virus Genotypes and Dermatoses in Penile Carcinoma in a Low Incidence Area in Europe.
LAB INVEST. 2011; 91: 884- [Poster]

Mannweiler S, Sygulla S, Winter E, Razmara Y, Pummer K, Regauer S Distribution of human papilloma virus genotypes and dermatoses in penile carcinoma.
Der Pathologe. 2011; 32, Suppl. 1: 114-114.-95. Jahrestagung der Deutschen Gesellschaft für Pathologie; JUN 16 - 19, 2011; Leipzig, GERMANY. [Poster]

Sygulla, S; Mannweiler, S; Tsybrovskyy, O; Razamara, Y; Pummer, K; Regauer, S Clinical relevance of the new TNM (2009) classification system for penile cancers: Is there a difference in lymphatic invasion and survival between pT1a and pT1b penile cancer?
Der Urologe. 2011; Suppl. 1(50):-63. Kongress der Deutschen Gesellschaft für Urologie; Sept 14 - 17, 2011; Hamburg, Germany. [Poster]

Sygulla S, Mannweiler S, Tsybrovskyy O, Razmara Y, Regauer S 2009 TNM classification for penile pt1 carcinoma: correlation with clinical outcome.
Der Pathologe. 2011; 32, Suppl. 1: 114-114.-95. Jahrestagung der Deutschen Gesellschaft für Pathologie; JUN 16 - 19, 2011; Leipzig, GERMANY. [Poster]

2010

Razmara, Y; Sygulla, S; Mannweiler, S; Pummer, K; Regauer, S Nachweis von HPV-16-Genotyp in penilen Plattenepithelkarzinomen
Der Urologe. 2010; Band 49: 104-105.-62. Kongress der Deutschen Gesellschaft für Urologie e.V.; 24.09.2010; Düsseldorf. [Poster]

Regauer, S; Sygulla, S; Mannweiler, S; Pummer, K; Razmara, Y Distribution of HPV genotypes in penile squamous cell carcinomas.
Abstract-CD 19th Congress of the European Academy of Dermatology and Venerology2010; 00118--Gotenburg 2010. 19th Congress of the European Academy of Dermatology and Venerology; October 6 - 10, 2010; Gotenburg, SWEDEN. (ISBN: 978-3-940017-69-7) [Oral Communication]

Sygulla, S; Mannweiler, S; Razmara, Y; Pummer, K; Regauer, S Distribution of HPV genotypes in penile squamous cell carcinomas.
ÖGP Konferenzband2010; -Kongress der ÖGP 2010; MAR 6, 2010; Graz, AUSTRIA. [Poster]

Sygulla, S; Mannweiler, S; Razmara, Y; Pummer, K; Regauer, S Verteilung von HPV-Genotypen in Platte-nepithelkarzinomen des Penis.
Journal für Urologie und Urogynäkologie.2010; 17, Sonderheft 3: 36--36. Gemeinsame Tagung der Bayerischen Urolgenvereinigung und der Österreichischen Gesellschaft für Urologie und Andrologie; JUN 10 - 12, 2010; Munich, GERMANY. [Oral Communication]

PANNONIA CONGRESS OF PATHOLOGY

GRAZ, MARCH 3-6, 2010

SPRING CONGRESS OF THE AUSTRIAN, CROATIAN, HUNGARIAN AND SLOVENIAN SOCIETIES OF PATHOLOGY AND IAP DIVISIONS

POSTER AWARD

for **Stephan Sygulla**

for his presentation

„Distribution of HPV genotypes in penile squamous cell carcinomas"

Univ. Prof. Dr. H. Popper
President of the Austrian Society of Pathology

Prof. Dr. József Tímár
President of the Hungarian Society of Pathology

Dr. Izidor Kern
President of the Slovenian Society of Pathology

Prof. Dr. Damir Babic
President of the Croatian Society of Pathology