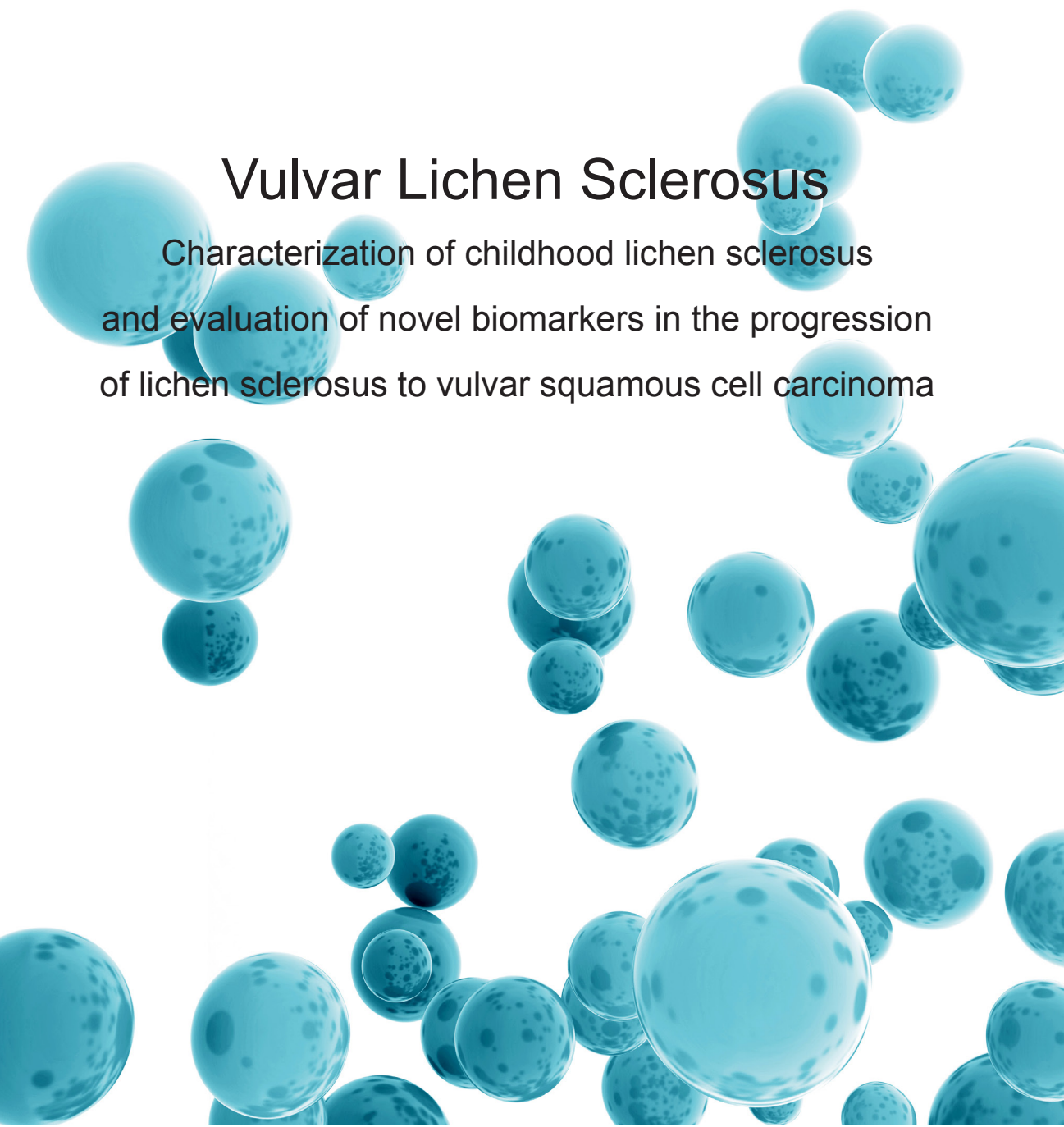
Acta Universitatis Tamperensis 2345

MARIA LAGERSTEDT

Vulvar Lichen Sclerosus

Characterization of childhood lichen sclerosus and evaluation of novel biomarkers in the progression of lichen sclerosus to vulvar squamous cell carcinoma



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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Errata

Vulvar lichen sclerosus

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Tiivistelmä, Tulokset;

> … ja valtaosassa vSCC näytteistä (46/53, 88%) havaittiin terveeltä iholta puuttuva Serpin A1:n värjäytyminen.
>
> **Pitäisi lukea**; … ja valtaosassa vSCC näytteistä (**35/40**, 88%) havaittiin terveeltä iholta puuttuva Serpin A1 värjäytyminen.

Abstract, Results;

> … and the majority (46/53, 88%) of the vSCC samples overexpressed Serpin A1.
>
> **Should read**; …and the majority (**35/40**, 88%) of the vSCC samples overexpressed Serpin A1.

Page 55: Tumour cell-specific Serpin A1 was equal in LS-depenedent and LS-independent vSCC…

Should read; Tumour cell-specific Serpin A1 **overexpression** was equal in LS-dependent and LS-independent vSCC…

Page 71: p16 was not detected in LS, but a coexistence of LS and p16 positivity was not uncommon.

Should read; p16 **positivity** was not detected in LS, but a coexistence of LS and p16 positivity **in vSCC patients** was not uncommon.

TIIVISTELMÄ

Tausta: Valkojäkälä eli lichen sclerosus (LS) on ihosairaus, joka ilmenee useimmiten sukuelinten ja peräaukon alueella vaaleina helposti haavautuvina ihomuutoksina. Valkojäkälän tavallisia oireita ovat kutina, kirvely ja kipu sekä ulostamis- ja virtsaamisvaikeudet, aikuisilla myös seksuaalielämän häiriöt. Valkojäkälään voivat sairastua sekä naiset että miehet, ja se voi puhjeta missä iässä tahansa. Lapsuusiässä sen esiintyvyys on 1:1000 ja aikuisilla 1:300. Naisilla ilmaantuvuudessa on kaksi huippua, ennen murrosikää ja vaihdevuosi-iän jälkeen. Valkojäkälä on autoimmuunisairaus, johon liittyy kohonnut muiden autoimmuunisairauksien riski. Valkojäkälä on pitkäkestoinen, taudinkuvaltaan aaltoileva ja vaikeasti ennustettava sairaus, joka voi johtaa genitaalialueen arpeutumiseen tai syöpään.

Ulkosynnyttimien eli vulvan levyepiteelisyöpä (vSCC) on harvinainen gynekologinen syöpä, johon Suomessa sairastuu vuosittain noin 130 naista. Valkojäkälä on merkittävä vulvan levyepiteelisyövän riskitekijä. Toinen keskeinen riskitekijä vulvan SCC:lle on korkean riskin papillooma-viruksen (hrHPV) kantajuus. Nämä kaksi etiologista tekijää syövän taustalla voidaan erottaa immunohistokemiallisten tutkimuksien avulla. Immunohistokemiallisen p53 värjäyspositiivisuuden tiedetään liittyvän valkojäkälään ja sen pohjalta syntyvään vSCC:hen, p16 positiivisuus puolestaan liittyy hrHPV:n pohjalta syntyneeseen vSCC:hen.

Estrogen related -reseptorit (estrogen related receptors, ERRs) ja Serpin A1 eli Alfa-1 antitrypsiini ovat huonon ennusteen merkkiaineita useissa syöpätyypeissä. ERR proteiinit, erityisesti ERRα, ovat solujen energia-aineenvaihdunnan säätelijöinä merkittävässä roolissa mm. immuniteetin säätelyssä ja kasvainten synnyssä. Serpin A1 osallistuu moniin fysiologisiin prosesseihin, kuten tulehduksen rajoittamiseen, toisaalta sillä on monia kasvaimen syntyyn vaikuttavia ominaisuuksia. Näitä merkkiaineita ei ole aikaisemmin vulvan levyepiteelisyövässä tutkittu.

Tavoitteet: Tutkimuksella haluttiin selvittää lapsuusiässä alkavan valkojäkälän liitännäissairastuvuutta, suvuttaista esiintymistä, vaikutuksia elämänlaatuun, komplikaatioiden esiintymistä ja malignisoitumisriskiä.

Koska valkojäkälän taudinkulku on arvaamaton eikä kliinisessä käytössä ole merkkiaineita yksillöllisen ennusten tarkentamiseen, halusimme selvittää onko aikaisemmin muissa syöpätyypeissä tunnistetuilla ennustetekijöillä ERRα, ERRβ, ERRγ, Serpin A1, p53 ja p16 osuutta valkojäkälässä, vulvan syövän esiastemuutoksissa ja vulvan SCC:ssä.

Aineisto ja menetelmät: Lapsuusiän valkojäkälää koskeva rekisteri- ja kyselytutkimus käsitti Tampereen yliopistollisessa sairaalassa vuosina 1981-2011 hoidetut alle 19-vuotiaat tytöt. Heistä vapaaehtoiset kutsuttiin ihotautilääkärin ja nuorisogynekologin tutkimusvastaanotolle. Kliinisen tutkimuksen ja elämänlaatukyselyn lisäksi heistä kerättiin verinäytteet autoimmuunitautien seulontaa ja mikrobiologisia tutkimuksia varten. Immunohistokemiallisia tutkimuksia tehtiin valkojäkälä- ja vSCC- potilaiden diagnostisista biopsianäytteistä ja vSCC potilaiden vulvektomianäytteistä, ja tuloksia verrattiin vapaaehtoisten luovuttamiin terveen vulvan ihon näytteisiin. ERRα:n, ERRβ:n ja ERRγ:n ilmentymistä tutkittiin kaikkiaan 120 potilaan 203 näytteestä, joista 110 oli valkojäkälä-, 6 syövän esiaste- ja 50 vSCC- näytteitä. Serpin A1:n, p53:n ja p16:n ilmentymistä tutkittiin 74 potilaan 120 näytteestä, joista 53 oli valkojäkälä-, 9 syövän esiaste- ja 40 vSCC -näytteitä. Serpin A1 seerumipitoisuuksia arvioitiin 84:n valkojäkälä-, vSCC- ja kontrollipotilaan näytteistä.

Tulokset: Rekisteritutkimus löysi 44 lapsuusiässä valkojäkälään sairastunutta tyttöä, joista 15 vastasi kyselyyn ja 12 osallistui tutkimusvastaanotoille. Lasten valkojäkälä osoittautui huonosti tunnistettuksi sairaudeksi, jossa autoimmuunisairauksien liitännäissairastavuus oli kohonnut. Valkojäkälän komplikaationa ilmeneviä vulvan alueen rakennemuutoksia ilmeni lähes viidenneksellä (n=8/44) potilaista. Lapsuusiän valkojäkälällä oli huomattava vaikutus elämänlaatuun.

ERRα, ERRβ ja ERRγ ilmenivät aina terveellä vulvan iholla. ERRα:n ilmenemisen havaittiin vähenevän 58%:ssa valkojäkälänäytteistä (64/110). ERRα värjäytyvyyttä ei havaittu tai se oli vähäistä kaikissa 50:ssä vulvan syöpänäytteessä. Serpin A1:n ilmeneminen lisääntyi syöpäprosessin edetessä, ja valtaosassa vSCC näytteistä (46/53, 88 %) havaittiin terveeltä iholta puuttuva Serpin A1:n värjäytyminen. Serpin A1 ilmeni syöpänäytteissä riippumatta siitä, edelsikö syövän syntyä valkojäkälän vai hrHPV:n aiheuttama tulehdus. Serpin A1 tai ERRα ei

ennustanut syöpäriskiä valkojäkäläpotilailla eikä osoittanut ennusteellista arvoa vSCC potilailla. p53:n ilmeneminen valkojäkälänäytteissä oli tavallista, mutta sitä esiintyi useammin valkojäkälässä, joka ei edennyt syöväksi (93 vs. 35%). Valkojäkäläpotilaiden syöpänäytteissä havaittiin hrHPV:hen liittyvä p16 positiivisuus 13 %:ssa (3/24) näytteistä.

Johtopäätökset: Lapsuuiän valkojäkälän oireet ja komplikaatiot aiheuttavat huomattavaa elämänlaadullista haittaa. Vulvan rakennemuutosten estämiseksi potilaiden riittävän tehokas alkuvaiheen hoito sekä pitkä ylläpitohoito ja seuranta ovat tärkeäitä. Kohonnut muiden autoimmuunisairauksien riski on huomioitava valkojäkäläpotilaiden seurannassa.

Muutokset ERRα:n and Serpin A1:n ilmentymisessä liittyvät sekä valkojäkälään, vulvan SCC:n esiastemuutoksiin että vSCC:hen. Nämä uudet merkkiaineet vahvistavat kroonisen tulehduksen olevan merkittävä tekijä syövän synnyssä ja tuovat uutta tietoa valkojäkälän ja vSCC:n tautimekanismista, mutta eivät vaikuta ennustavan syöpäriskiä valkojäkäläpotilailla tai liittyvän vSCC:n ennusteeseen. Myös p53 proteiinin tärkeä rooli vSCC:ssa vahvistui. Osalla vSCC-potilaista esiintyi riskitekijöistä sekä p16 positiivisuus että valkojäkälä, mikä kannustaa valkojäkäläpotilaiden HPV-kantajuuden tutkimiseen.

ABSTRACT

Background: Lichen sclerosus (LS) is a dermatitis typically affecting the genital and perianal area with pearly white and atrophic lesions. LS symptoms include itching and a burning sensation, as well as pain, dysuria and constipation- and, in adults, problems in the sexual life. LS has a considerable effect on the patients' quality of life (QoL). LS can occur in women and men, and at any age. In childhood, the prevalence is approximately 1:1000 and in adulthood 1:300. In the female population, the incidence is dual-peaked, being highest in prepubertal girls and postmenopausal women. LS is an autoimmune disease with an increased risk of other autoimmune diseases. The course of LS is chronic, relapsing and remitting, and it may lead to scarring and a disturbance in the vulvar architecture or a progression to malignancy.

Vulvar squamous cell carcinoma (vSCC) is a rare gynaecological malignancy. The incidence in Finland is ca. 130 women per year. LS is an important risk factor for vSCC. The other main risk factor for vSCC is a high-risk human papilloma virus (hrHPV) infection. Immunohistochemical (IHC) studies of tumour suppressor proteins p53 and p16 can be used to discriminate these two aetiological pathways. p53 positivity in IHC is associated with LS- and LS-dependent vSCC, while p16 positivity is associated with hrHPV-dependent vSCC.

Oestrogen related receptors (ERRs) and Serpin A1 (also called alfa-1 antitrypsin) are markers of a poor prognosis in several cancer types. ERR proteins, especially ERRα, are master regulators of the energy metabolism in cells and thus play a role in, for example, tumorigenesis and immunology. Serpin A1 participates in many physiological processes, such as restricting the inflammatory response, but it also has tumorigenetic properties. These markers have not been previously studied in LS or vSCC.

Aims: The current study investigated the co-morbidity, family history, impact on the QoL, as well as the complications and the risk of malignancy in childhood-onset LS. Since the course of LS is unpredictable and there are no markers in clinical practice to predict the risk of malignancy in an individual patient, we set out

to study the role of these previously identified tumour markers − ERRα, ERRβ, ERRγ, Serpin A1, p53, and p16 − in LS, vulvar premalignant lesions and vSCC.

Materials and methods: The register study and questionnaire included childhood-onset LS patients less than 19 years of age treated at Tampere University Hospital in 1981-2011. The register study found 44 childhood-onset LS patients. The patients were invited to a clinical examination by a dermatologist and a paediatric gynaecologist. The visit also included QoL questionnaires and the collection of blood samples for the screening of other autoimmune diseases and microbiological aetiologies. Immunohistochemical studies on normal vulvar skin samples from volunteers, diagnostic biopsy samples from LS and vSCC patients, and vulvectomy samples from vSCC patients were obtained. The ERRα, ERRβ and ERRγ expressions were studied in 203 samples from 120 patients: 110 LS samples, 6 vulvar premalignant lesion samples and 50 vSCC samples. The studies on Serpin A1, p53, and p16 included 120 samples from 74 patients: 53 LS, 9 vulvar premalignant lesions and 40 vSCC samples. Serpin A1 serum concentrations were analysed from 84 control, LS, and vSCC patients.

Results: The register study found 44 childhood-onset lichen sclerosus patients, 15 of whom completed the questionnaire and 12 attended the clinical visits. Childhood LS turned out to be a challenging diagnosis for clinicians. The risk of other autoimmune diseases was elevated. Vulvar scarring and architectural changes of the vulva occurred in almost every fifth of patient (8/44). The impact on the QoL in LS was significant.

Normal vulvar skin expressed ERRα, ERRβ and ERRγ. A decrease in ERRα staining was detected in 58% of the LS samples (64/110). A complete loss of or a substantial decrease in ERRα expression occurred in all 50 vSCC samples. The expression of Serpin A1 increased in a malignant progression to vSCC, and the majority (46/53, 88%) of the vSCC samples overexpressed Serpin A1. The overexpression of Serpin A1 was associated with vSCC in both LS-dependent and HPV-dependent vSCC cases. Neither of the studied markers predicted the risk of cancer in LS patients or had a correlation with the overall survival of vSCC patients. Positivity for p53 in LS samples was common, and LS without a malignant progression was p53-positive more often than LS with a malignant progression (93% vs. 35%). In LS-dependent vSCC, p16 positivity was detected in 13% (3/24) of the patients.

Conclusions: The symptoms and complications of childhood LS have a significant effect on the QoL of the patients. To prevent complications from LS, initial treatment must be effective, and the maintenance therapy and follow-up long enough. The risk of other autoimmune diseases should be kept in mind in the surveillance.

A decrease in ERRα expression and an increase in Serpin A1 expression are associated with vSCC in both the LS- and the HPV-related pathways. These novel biomarkers add knowledge of these inflammatory pathways to vSCC and shed light to the pathogenesis of vSCC, but seem to lack prognostic value in LS or vSCC patients. The essential role of p53 in vSCC was established. p16 positivity also occured in LS-dependent vSCC. Therefore, testing for hrHPV types in LS patients is important.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on original publications, which are referred to by their Roman numerals (I-III).

I. Lagerstedt M*, Karvinen K*, Joki-Erkkilä M, Huotari-Orava R, Snellman E, Laasanen SL. Childhood lichen sclerosus- A challenge for clinicians. Pediatric Dermatology 2013; 30: 444-50.

* equal participation

II. Lagerstedt M, Huotari-Orava R, Nyberg R, Mäenpää JU, Snellman E, Laasanen SL. Reduction in ERRα is associated with lichen sclerosus and vulvar squamous cell carcinoma. Gynegologic Oncology 2015; 139: 535-40.

III. Lagerstedt M, Huotari-Orava R, Nyberg R, Nissinen L, Farshchian M, Laasanen S-L, Snellman E, Mäenpää JU, Kähäri V-M. Tumor-cell specific Serpin A1 expression in vulvar squamous cell carcinoma. Submitted.

ABBREVIATIONS

CDKN2A	Cyclin-dependent kinase inhibitor 2A
DLQI	Dermatology life quality index
dVIN	Differentiated vulvar intraepithelial neoplasia
EBV	Epstein-Barr virus
EDF	European Dermatology Forum
EGFR	Epidermal growth factor receptor
ERR	Oestrogen related receptor
FIGO	International Federation of Gynaecology and Obstetrics
HE	Hemtoxylin and eosin
HSIL	High-grade squamous intraepithelial lesion
hrHPV	High-risk human papilloma virus
HRAS	Harvey rat sarcoma viral oncogene homologue
HRQoL	Health-related quality of life
IFR6	Interferon regulatory factor 6
IHC	Immunohistochemistry
IL-1RA	Interleukin-1 receptor antagonist

LS	Lichen sclerosus
MGMT	O-6-Methylguanine-DNA Methyltransferase
PDT	Photodynamic therapy
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pRB	Retinoblastoma protein
PRP	Platelet-rich plasma
PTEN	Phosphatase and tensin homologue
p14	Tumour suppressor protein 14
p16	Tumour suppressor protein 16
p53	Tumour suppressor protein 53
RASSF2	RAS-association domain family member 2
Rhoa	RAS homologue gene family A
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1
ROS	Reactive oxidative species
SCC	Squamous cell carcinoma
Serpin A1	Serpin peptidase inhibitor clade A member 1 protein
TNFα	Tumour necrosis factor α
Tp53	Tumour protein 53 gene
vSCC	Vulvar squamous cell carcinoma

QoL	Quality of life
15D	15-dimensional questionnaire on quality of life

1 INTRODUCTION

Lichen sclerosus (LS) is a chronic, autoimmune dermatosis which can affect women and men at any age (Fistarol et al. 2013). The typical presentation of LS involves pearly white, sometimes erosive and fragile plauques the skin of the genital area (Powell et al. 1999). Childhood-onset LS is rare and clinically variable, which may cause a delay in diagnosis (Powell et al. 2001). The peak of incidence in females occurs in times of low oestrogen production, in prepuberty and postmenopause, which has raised a question of role of sex hormones in LS. The majority of LS patients suffer from intense itching and a burning sensation, difficulties in urinating and defecating and a disturbance of the sexual life (Powell et al. 1999). LS has remarkable effects on the patients' quality of life (QoL) (Lansdorp et al. 2013, van Cranenburgh et al. 2017). However, QoL in childhood LS has not been studied previously. The complications of LS include scarring and alterations of the vulvar architecture, and, in approximately 2%-5% of LS patients, the disease progresses to malignancy (Fistarol et al. 2013, Halonen et al. 2017).

Vulvar squamous cell carcinoma (vSCC) is a rare gynecologic malignancy with 130 new cases in Finland annually (Finnish Cancer Registry 2017). The incidence of vSCC has risen slightly in the past decades, particularly in younger age groups, which is likely due to HPV infections (Lavarato-Rocha 2016, Finnish Cancer Registry 2017, Wakeham et al. 2017). However, vSCC is typically a cancer of elderly women. In the Western countries, the overall five-year survival of vSCC lies around 70% (del Pino et al. 2013).

Two distinct aetiological routes can lead to vSCC. Depending on the study population, hrHPV types are detected in 20%-70% of vSCC patients (del Pino et al. 2013, Cheng et al. 2016). LS can be found in 30%-40% of vSCC patients (Davick et al. 2017). The exact pathogenetic mechanisms leading to vSCC remain unknown, and the studies on biomarkers predicting the malignant potential of LS have yielded contradictory results (Carlson et al. 2013).

The aim of this study was to gain more insight into a rare entity of childhood-onset LS, and to define its course, comorbidities and, complications, as well as the impacts on the QoL of the patients. The other aim was to clarify the pathogenetic mechanism in the malignant transformation to vSCC by evaluating new

biomarkers, oestrogen related receptors (ERRs) and Serpin A1, in LS, in premalignant lesions and in vSCC. In addition, we wanted to compare Serpin A1 to the two most widely studied biomarkers in vSCC, p53 and p16. We also set out to evaluate the value of these markers in predicting the prognosis of LS and vSCC.

2 REVIEW OF THE LITERATURE

2.1 Lichen Sclerosus (LS)

2.1.1 Epidemiology of lichen sclerosus

Lichen sclerosus (LS) is a chronic autoimmune dermatitis which has been reported in all ages and both sexes (Powell et al. 1999). The estimate female-to-male ratio varies between 1:1 and 10:1, depending on the study population (Powell et al. 1999, Becker 2011, Fistarol et al. 2013). In the female population, the incidence is dual-peaked in times of low oestrogen production: before menarche (onset at 4-6 years of age on average) and after menopause (Powell et al. 2001, Higgins et al. 2012). The prevalence of LS is estimated to be 1:300 in postmenopausal women and 1:1000 in prepubertal girls (Kirtching 2016). However, LS patients are often undiagnosed: patients with no symptoms, patients whose diagnosis is delayed or false, or those who are too embarrassed to seek help (Powell et al. 1999). Caucasian ethnicity dominates in LS (Funaro 2004).

2.1.2 Aetiology of lichen sclerosus

Genetic factors

A positive family history of LS is found in 17% of paediatric and in 12% of adult LS patients (Powell et al. 2001, Fistarol et al. 2013). Several studies report sibling pairs, heterozygote and monozygote twin sisters, as well as mothers and daughters, indicating a heritable predisposition to LS (Aslanian et al. 2006, Liu et al. 2015). Germ-line mutations specific to LS have not been identified.

In first-degree relatives of LS patients, autoimmune disorders are found in 17%-65% of the cases, and a positive family history of other autoimmune diseases is more frequent in childhood-onset than adult-onset LS (Powell et al. 2001, Aslanian et al 2006). The human leukocyte antigen (HLA) system is closely associated with

numerous autoimmune diseases (Liu et al 2015). HLA polymorphism associates with LS according to several studies, but the associated HLA subtype varies between different geographical regions and etnicities (Liu et al. 2015). HLA DQ7, HLA DQ8 and HLA DQ9 are more frequent in LS patients than in healthy controls, and they have been found in 78% of studied patients, in comparison to 40% of the controls (Funaro 2004, Fistarol et al. 2013). Polymorphism of the *interleukin-1 receptor antagonist (IL-1 RA)* has also been associated with LS (Clay et al. 1994).

Local factors

Infections have been studied as a trigger for LS (Powell et al. 1999). The role of a *Borrelia bungdorferi*-infection in LS is controversial. Several cases with a concomitant LS and *Borrelia* infection have been reported, as has been the improvement of LS symptoms after the treatment of the *Borrelia* infection (Zollinger et al. 2010). The detection of *Borrelia* DNA by means of PCR has ranged from 0%-100% depending on the study and geographical area, i.e. approximately 34% in Europe (28/83 cases). However, even in endemic areas, *Borrelia* has been detected in only 2% of LS cases (Zollinger et al. 2010). The detection of *Borrelia* by serological, cell culture, or even PCR methods is difficult, which may affect these results (Fistarol et al. 2013). When focal-floating microscopy is used for the detection of *Borrelia* remarkably higher numbers are found: *Borrelia* was found in 63 % (38/60) of LS samples, and even some PCR-negative skin biopsy samples turned out to be positive for *Borrelia* (Fistarol et al. 2013). The *Epstein-Barr virus (EBV)* has also been studied in connection with LS. *EBV* DNA was found in 26.5 % of 34 vulvar biopsies of patients with LS as opposed to 0% in controls (Fistarol et al. 2013). Genital infections, usually caused by *Escherichia coli* or *streptococci*, are common in LS patients, but they are considered more secondary infections than triggering factors (Powell et al. 1999). LS patients are also more susceptible to yeast infections such as *Candida albicans* or fungal infections caused by, for example *Trichophytom* or *Microsporum* species, but mycosis of the vulva is considered more a secondary phenomenon than a risk factor (Kirtschig et al. 2015, Day et al. 2016).

LS can be triggered by constant friction or trauma, which is called the Köbner phenomenon (Todd et al. 1994). LS can also reoccur in grafts or vulvectomia and circumcision scars (Poindexter et al. 2007). In children, traumas caused by sexual abuse may influence the onset of LS by the Köbner phenomenon (Warrington et al. 1996).

Hormonal factors

Due to the highest prevalence of LS occuring in times of low oestrogen production, there has been an attempt to clarify the role of sex hormones in the etiology of LS. The role of estrogen has remained ambiguous. No association with pregnancy, hysterectomy or hormone-replacement therapy has been found. Treatment with topical or oral oestrogen has also proven inefficient (Powell et al. 1999).

Decreased serum 5α-reductase activity has been shown to associate with LS (Friedrich et al. 1984).

A loss of androgen receptors within LS progression has also been detected (Clifton et al. 1999). However, topical testosterone has shown no benefit in the treatment of LS (Powell et al. 1999). It has also been suggested that anti-androgenic oral contraceptive pills are associated with early-onset adulthood vulvar LS (Kirtschig et al. 2015).

Turner syndrome has been associated with LS. A study of 133 French females with Turner's syndrome found prevalence of LS as high as 17%. This association may be explained by the low oestrogen levels or high prevalence of coincident autoimmune diseases in Turners syndrome (Chacktoura et al. 2014).

2.1.3 Immunology of lichen sclerosus

LS is an autoimmune disease with a T-helper type 1 (Th1) response (Terlou et al. 2012). Th1 response associates with autoimmunity and chronic inflammation. A key cytokine in Th1 response is IFNγ, which is found to be up-regulated in LS. Upregulation of IL-1α and IL18 has also been reported (Terlou et al. 2012).

A study of 350 women with LS revealed that 21.5 % of the patients had at least one additional autoimmune disease (Fistarol et al. 2013). In girls with LS, 7%-14% have another autoimmune disease (Helm et al.1991, Powell et al. 2001, Oyama et al. 2003, Maronn et al. 2005). The most common related autoimmune diseases are autoimmune thyroiditis (12 %), alopecia areata (9 %), vitiligo (6 %), and pernicious anaemia (2 %) (Funaro 2004).

Circulating IgG autoantibodies to extracellular matrix protein 1 (ECM-1) have been detected in 74% of female LS patients compared to 7% of controls (Oyama et al. 2003). EMC-1 affects keratinocyte differentiation and controls the structure of the dermis and basement membrane. Loss-of-function mutations in the

ECM1 gene cause lipoid proteinosis (OMIM 247100), also known as the Urbach-Wiethe disease or hyalinosis cutis et mucosae (Chan et al. 2007). This recessive disease is characterized by skin and mucosal infiltrations, thickening and scarring. Histologically most striking feature is the deposition of hyaline material in the dermis, which is a common feature in LS (Chan et al. 2007).

Antibodies targeting the basement membrane zone (BP180 and BP230) have been found in one-third of the patients with vulvar LS and in almost half of paediatric LS patients (Baldo et al. 2010, Fistarol et al. 2013). However, the most recent studies found elevated anti-BMZ in only 3%-6% of LS patients, compared to 94% of pemphigus bullosus patients (Gambichler et al 2011, Patsatsi et al. 2014). Therefore, the role of basement membrane zone antibodies in LS remains open.

MicroRNA-155 (miR-155) is up-regulated in LS (Terlou et al. 2012). MiRs are small fragments of non-coding RNA that modify gene expression post-transcriptionally. MiR-155 has an important role in many autoimmune diseases (such as multiple sclerosis and rheumatoid arthritis) and in chronic inflammation. MiR-155 upregulation affects regulatory T cells (Treg) by altering their suppressing function to CD4+ T cells which results in an impaired immune reaction, a Th1 response and susceptibility to an autoimmune response (Terlou et al. 2012).

2.1.4 Histopathology of lichen sclerosus

The most distinctive histological feature in LS is the homogenization of collagen to a pale-staining zone in the upper dermis, with inflammatory infiltrates underneath (Powell et al. 1999) (Figure1). The inflammatory changes include CD4- and CD8-positive lymphocytes, macrophages and mast cells. The presence of mast cells could explain the pruritus and also the extracellular matrix changes (Powell et al. 1999). The hydropic degeneration and vacuolar changes of the basal layer, as well as hyperkeratosis or atrophy of the epidermis are seen. Structural changes in collagen and a diminished amount of elastin and fibrillin fibres are detected in the oedematous dermis with deposits of hyaluronic acid and dermatan sulfate proteoglycan (Powell et al. 1999, Funaro 2004). Squamous hyperplasia has been reported in up to one-third of patients with LS and is often caused by scratching (Funaro 2004).

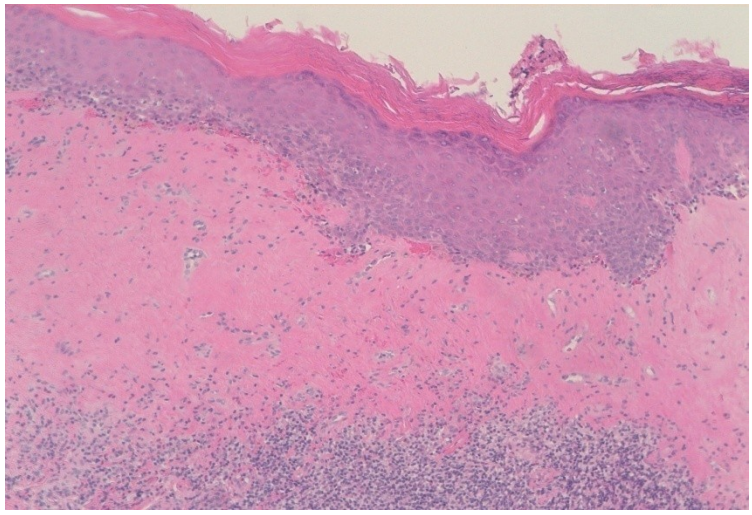

Figure 1. Typical histology of lichen sclerosus: hyperkeratosis and vacuolar changes in the epidermis, a homogenization of collagen and lymphocyte infiltrate in the dermis. *Hematoxylin and eosin (HE) stain, original magnification x100*. Reprinted from Pediatric Dermatology, 30, Lagerstedt M, Karvinen K, Joki-Erkkilä M, Huotari-Orava R, Snellman E, Laasanen SL. Childhood lichen sclerosus- A challenge for clinicians, 444-50 (Copyright 2013), with permission from Wiley and sons.

2.1.5 Clinical features of lichen sclerosus

In women and girls, LS most often affects the anogenital area in a figure-of-eight-like pattern. A minority (6 %) of LS patients have extragenital lesions only, and in 11%-15% of the patients, LS lesions are located in both the genital and the extragenital area (Powell et al. 1999, Cooper et al 2004). Extragenital lesios are typically located in the inner thigh, the submammary area, the wrists or shoulders (Fistarol et al 2013). The clinical picture of LS lesions can vary, and this can be challenging to clinicians. The typical presentation involves polygonal papules with a central indentation, which coalesce over time and form pearly-white, fragile plaques. Oedema, purpura, erosions, fissures or submucosal haemorrhage can also be seen (Cooper et al 2004). In contrast to lichen planus, genital mucosal involvement does not occur, the vagina and cervix always being spared (Fistarol et al. 2013).

Pruritus is the most common symptom in females (Cooper et al. 2004). Itching, a burning sensation, dysuria and pain in connection with defecation also exist. Constipation due to painful fissures may occur. LS may lead to vulvodynia and

dyspareunia (Powell et al. 1999, Smith et al 2009). LS can also be asymptomatic. As many as up to 39% of adult LS patients are symptomless, but the majority of paediatric LS patients are symptomatic (Fruchter et al. 2017).

2.1.6 Impact of lichen sclerosus on the quality of life

The quality of life (QoL) can be measured in patients with dermatological diseases by using structured questionnaires. The most commonly used dermatological disease-specific instrument, the Dermatology Quality of Life Index (DLQI), includes ten questions measuring the QoL (van de Niuewenhof 2010). The questions are related to difficulties in the treatment of dermatosis, as well as symptoms, social aspects and restrictions in the working, social and sexual life (Finlay et al. 1994). The Skindex-29 is another dermatology-specific tool, but as it consisting of 29 items, it is mainly utilized for study purposes and not in general practice (Lansdorp et al. 2013). The Health-related quality of life 15D (HRQoL, D15) is a 15-dimensional generic, standardized questionnaire for adults, which can be used in measuring the HRQoL in any disease. It assesses mobility, vision, hearing, breathing, sleeping, eating, speech, excretion, usual activities, mental function, discomfort and symptoms, depression, distress, vitality, and sexual activity. The questionnaire has been thoroughly validated for psychometric properties in studies in several countries (Sintonen 2011). In paediatric patients with dermatological disease, the Children's Dermatology Life Quality Index (CDLQI) can be used (Olsen et al. 2016). No specific tool exists for measuring QoL in LS patients.

LS is claimed to have a strong impact on the QoL. LS lesions are typically located in intimate areas, which may cause shame, fear and embarrassment (Powell et al. 1999). Two studies with Dutch female LS patients using the Skindex-29 found a moderately impaired quality of life: compared to patients with psoriasis, hand eczema, acne vulgaris and even neurofibromatosis, the QoL in LS patient was worse, which is consistent with high burden of the disease (Lansdorp et al. 2013, van Cranenburgh et al. 2017). In adults, LS especially interferes sexual life (van de Niuewenhof 2010). There are no previous studies on the QoL in peadiatric LS patients.

2.1.7 Diagnosis of lichen sclerosus

The diagnosis of LS can be based on the clinical picture, but a biopsy is often taken to confirm the diagnosis (van de Niuewenhof et al. 2008). With the typical clinical picture, the diagnosis can also be confirmed by means of dermatoscopy: recent LS lesions are characterized by homogeneous whitish areas surrounded by an erythematous halo, with the presence of yellow circles (follicular pseudo-apertures – comedo-like openings), which complies with the histopathologic features of ortohyperkeratosis and follicular plugging. Older lesions present with no halo (Nobrega et al. 2016).

The differential diagnoses of LS in girls are infections, allergic or irritative dermatitis, vitiligo and lichen planus, as well as trauma or sexual abuse (Poindexter et al. 2007). In females, the differential diagnoses also include bullous dermatosis such as bullous pemphigoid or intraepitheal neoplasia (Fistarol et al. 2013). Morphea and extragenital lichen sclerosus can also co-exist, and the differential diagnosis of these two entities can be difficult (Kreuter et al. 2012). Previously, these were considered as different manifestations of the same entity due to similarities in the clinical picture and histology (Nobrega et al. 2016). Vulvar lichen planus and LS can also manifest in the same patient (Day et al. 2017).

The average age of LS onset is 4-6 years in girls and 60 years in adult females. The delay in diagnosis is remarkable, 1-2 years in girls and 5 years in adult females on average (Berthe-Jones et al. 1991, Ridley et al. 1993, Powell et al. 2001, Cooper et al. 2004, Maronn et al. 2005, Smith et al. 2009, Patrizi et al. 2010, Jensen et al. 2012, Fistarol et al. 2013).

2.1.8 Prognosis and complications of lichen sclerosus

Previously it was thought that childhood lichen sclerosus dissolves in puberty. However, studies of childhood LS have proven this wrong (Powell et al. 2001, Smith et al. 2009). In women and girls, LS is a chronic remitting and relapsing dermatitis (Cooper et al. 2004). Scarring and changes in the vulvar architecture are well-known complications of LS (Fistarol et al. 2013). The labia minora may be reabsorbed and fused or the clitoris buried under scar tissue, which is defined as clitoral phimosis. The vaginal introitus may become narrowed and pseudocysts may appear (Fistarol et al. 2013, Flynn et al. 2015).

The lifetime risk of vulvar SCC is 3%-5% in adult-onset LS (Halonen et al.2017, Fistarol et al. 2013). The risk of cancer in childhood-onset LS is unknown. There is

a case report of a childhood-onset LS patient who had vulvar SCC at the age 32 (Smith et al. 2009).

Treatment with potent topical corticosteroids seems to diminish the risk of architectural changes in the vulva and vSCC. A large study of 507 women with vulvar LS revealed that scarring occurred during follow-up in 3.4% of patients with adequate treatment and follow-up, while the risk was up to 40.0% in partially compliant patients. Vulvar SCC occurred in none of the compliant patients and in 7 (4.7%) of 150 non-compliant patients (Lee et al. 2015). In another large study with 327 women, a delay in diagnosis of 2 years or less was associated with less scarring (Cooper et al. 2004).

2.1.9 Markers predicting the malignant potential of lichen sclerosus

Due to the unpredictable course of LS, protein markers predicting the individual prognosis of LS patients are warranted for clinical use.

Protein markers predicting the malignant potential of LS have been studied, and immunohistochemistry of p53, KI-67 and p16 may be useful (Carlson et al. 2013). A recent review by Trietcsh et al. shows that *Tp53* mutations are detected in 70% of LS cases, unrelated to the prognosis of LS (Trietcsh et al. 2015). Mutations of *Tp53* lead to an altered p53 protein, which accumulates into cell nuclei and can be detected by immunohistochemistry (IHC) (Sighn et al. 2015). Several earlier studies have shown an association between long-standing LS with presumably higher risk of vSCC and increased p53 expression, but this has been debated: p53 expression can also be a sign of ischaemic stress (Sadalla et al. 2011). However, p53 immunopositivity is an early event in LS-dependent carcinogenesis, but its use as a progression marker is questionable (Carlson et al. 2013).

Strong "block-like" p16 immunopositivity associates strongly with hrHPV types, and it can therefore be used as a surrogate marker for hrHPV infection (Cheng et al. 2016). HPV infection in LS has previously been reported mainly in male patients in case series (Carlson et al. 2013). Hald et al. concluded in their review that taken together, the prevalence of HPV in male patients with LS is an estimated 29% and, in female LS patients 8%. However, studies in this field are limited (Hald et al. 2017). Therefore, the prognostic value of p16 in LS has not been established (Carlson et al. 2013). Notably, topical corticosteroid therapy may also reactivate both low-risk and high-risk HPV types in genital lichen sclerosus (von Krogh et al. 2002).

Ki-67, a nonhistone matrix protein, is commonly used as a marker of the cell proliferation rate (Carlson et al. 2013). Increased Ki-67 expression levels are detected in LS and vulvar SCC compared to normal vulvar skin, making it a potential progression marker in LS (Rolfe et al. 2001, Koymatsu et al. 2003, Raspollini et al. 2007). However, a study by Scurry et al. found no difference in Ki-67 levels between LS with and without cancer (Scurry et al. 1998).

2.1.10 Management of lichen sclerosus

Topical Treatment

According to the European Dermatology Forum (EDF) Evidence-based Guideline for LS, the treatment goal is, first of all, the relief of symptoms. However, the prevention of complications is also desirable. Ultrapotent topical corticosteroids are the treatment of choice for the management of LS. Initial treatment with an ultrapotent corticosteroid (clobetasol propionate 0.05%) is recommended daily for 3 months, with a possible reduction in frequency after 1 month in milder cases, then typically every other day after one month and two times a week for the last month (Kirtschig et al. 2015). Three months daily treatment with clobetasol propionate 0.05% has been found to relieve the symptoms in 75%-90% of patients (Bracco et al. 1993, Garzon et al. 1999). Complete reversal of the signs of LS occurred in 20% of adult women and in 20%-70% of girls (Cooper et al. 2004). Mometasone furoate 0.1% has also proven as effective and as safe as clobetasol furoate 0.05% (Virgili et al. 2014), and has shown no significant side effects in the long term (Bradford et al. 2010, Kirtschig et al. 2015). A randomized study by Borghi et al. compared treatment with mometasone furoate 0.1% applied either in 5 days a week or 5 days a week for the first 4 weeks, every other day for the following 4 weeks, and 2 days a week for another 4 weeks. These treatment regimens were equally effective, and approximately 80% of the patients responded to the treatment (Borghi et al. 2015).

Tacrolimus and pimecrolimus have also been used: in paediatric LS, 11/14 of patients had complete response in 10 months of treatment (twice daily for 4 months, then maintenance therapy if remission) with tacrolimuse 0.03% (Li et al. 2013). In adult LS patients 43% had a complete response and 34% a partial response with 3 months of tacrolimuse 0.1% use, and the effect was stronger in patients under 50 years of age (Hengge et al. 2006). Pimecrolimus 1% was more

effective, and complete remission was achieved in the 70% of patients after 3 months (Kirtschig et al. 2015). Concerns of the risk of malignancy have been associated with long-term use of tacrolimuse and pimecrolimus, but there is no data to support this association, and the EDF Guideline concludes that long-term use of tacrolimus and pimecrolimus is considered safe (Spergel et al. 2006, Kirtschig et al. 2015). However, the ultrapotent steroid ointments are more effective in the treatment of LS (Funaro et al. 2014).

For maintenance therapy a potent or ultrapotent corticosteroid or tacrolimuse/pimecrolimus can be utilized from 1-2 times per month to 1-3 times per week individually (Kirtschig et al. 2015). In girls with LS, the maintenance therapy is recommended to continue for two years after remission (Ellis et al. 2015). In adults, the optimal duration of maintenance therapy has not been established. Attention should also be paid in the avoidance of mechanical irritation and use of moisturizers (Kirtschig et al. 2015).

Steroid injections can be considered in patients with treatment-resistant itching or in patients who are unable to apply topical steroids (Mazdisnian et al. 1999). No controlled randomized studies exist on topical retinoids in vulvar LS, although there are studies showing a beneficial effect in some patients (Virgili et al. 1995). Topical oestrogen, testosterone or progesterone treatment has proven ineffective (Kirtschig et al. 2015).

UV- treatment, especially UVA can be used in extragenital LS. In vulvar LS, PUVA treatment is as effective as treatment with clobetasol propionate 0.05% and it can also be considered as the second line treatment with some patients (Terras et al. 2014). However, the increased risk of malignancy associated with LS itself and with UV-treatments has to be taken into consideration (Kirtschig et al. 2015).

Photodynamic therapy (PDT) has been studied in small case series of LS, and it is effective on pruritus but histologically and clinically the effect is poor. PDT is also painful for patients, and thus cannot be recommended (Ojelek et al. 2010).

Systemic treatment

If topical treatment fails, oral retinoids, a pulse steroid combined with low-dose metotrexate, cyclosporine or metotrexate can be used. None of the systemic treatments have a high level of evidence (Kirtschig et al. 2015). A small controlled trial showed that asitretin 20-30mg per day had an effect on vulvar LS symptoms, and the reversal of clinical signs was achieved in 64% of 24 LS patients (Bousema et al. 1994). Asitretin can therefore be tried if treatment with clobetasole

propionate fails (Kirtschig et al. 2015). Metotrexate with a dose of 10-15mg per week also seems to be effective in severe extragenital LS when used alone or in a combination with pulse-steroid (Kreuter et al. 2009). Only one study on cyclosporine exists where high doses (3-4mg/kg) of cyclosporine were given to 5 vulvar LS patients, with success in diminishing symptoms and signs (Bulbul et al. 2007). One case series on vitamin A and E has been published showing an effect on vulvar LS (Kirtschig et al. 2015). Platelet-rich plasma (PRP) is processed from the patients' own blood and is rich in growth factors, thus improving wound healing. Needling PRP to 28 vulvar LS patients resulted in the majority experiencing good effect on the clinical signs and symptoms, and the treatment can thus be considered as a promising new option for LS (Behnia-Willison et al 2016).

The architectural changes in the vulva, the narrowing of the introitus and clitoral phimosis, can be treated with surgical methods or CO2-laser (Kirtschig et al. 2015). Surgical treatment has been found to improve clitoral sensation in 75% and to decrease dysparenunia in 91% of LS patients (Flynn et al. 2015).

2.2 Premalignant vulvar lesions

2.2.1 Epidemiology and classification of premalignant vulvar lesions

The rates of premalignant lesions of the vulva have increased in the past few decades (van de Niuewenhof et al. 2008, Lavarato-Rocha 2016). On the basis of histological features, premalignant vulvar lesions have previously been subdivided into the usual vulvar intraepithelial neoplasia (uVIN) and differentiated vulvar intraepithelial neoplasia (dVIN) types (WHO 2003 classification). The 2014 WHO classification divides uVIN into low-grade squamous intraepitehelial lesions (LSIL), previously VIN1, and high-grade squamous cell intraepithelial lesions (HSIL), previously VIN2-3, while dVIN classification remained unchanged (Kurman et al. 2014). HSIL and LSIL associate with HPV: hrHPV types 16 and 33 are mainly found in HSIL and low-risk HPV types 6 and 11 in LSIL (Hoang et al. 2016). LSIL is not considered a real precursor lesion, and half of these infectious lesions resolve without any treatment (Lewis et al. 2017, Planner et al. 1988). dVIN associates with LS and other vulvar dermatoses (Hoang et al. 2016).

The peak of incidence in HSIL is at 35-39 years of age, while dVIN occurs in women at more advanced age (van de Niuewenhof et al. 2011). dVIN behaves as a

real carcinoma *in situ* with a considerable risk for progression (35% of dVIN vs. 3%-16% of HSIL) (Singh et al. 2015, Trietsch et al. 2015). For the development of HSIL several risk factors have been identified: the number of sexual partners, smoking and immunosuppression (del Pino et al. 2013, Hoang et al. 2016). Factors influencing the development of dVIN from LS are less well understood, but age and intensive inflammation can be considered risk factors for dVIN (van de Niuewenhof et al. 2009, Lee et al. 2015).

2.2.2 Histopathology of premalignant vulvar lesions

The histological features of HSIL are easily identified: the epidermis is thickened and often shows hyperkeratosis. A loss of cell maturation, a high nuclear-to-cytoplasmic ratio, pleomorphism and numerous mitotic cells are seen (del Pino et al. 2013) (Figure 2A). dVIN in turn is a much more difficult histological diagnosis and often missed (Singh et al. 2015). The hallmark of dVIN is abnormal, atypical keratinocytes in the basal layer of epidermis with a maturation of superficial layers. Rete ridges are typically prominent (del Pino et al. 2013) (Figure 2B).

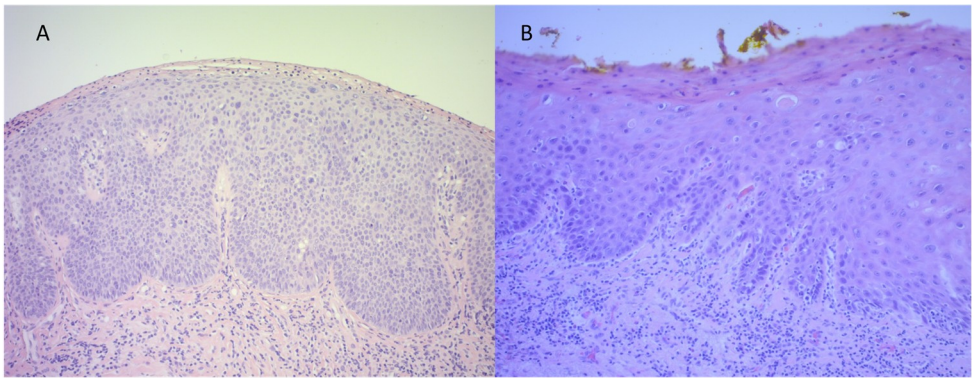

Figure 2. The histology of diffentiated vulvar intraepithelial neoplasia (dVIN) and high-grade squamous intraepithelial neoplasia (HSIL). A) The histology of HSIL shows a loss of maturation of keratinocytes and an expansion of immature basaloid cells on the surface of the epidermis, as well as dyskeratosis, nuclear pleomorphism and numerous mitoses. B) The histology of dVIN shows elongated rete ridges, basal atypia, dyskeratosis and atypical mitoses, with a normal maturation of keratinocytes in superficial layers. *HE staining, Original magnification x100.* Histological pictures by R.H-O.

2.2.3 Clinical features and treatment of premalignant vulvar lesions

The clinical picture of HSIL typically includes a macula, papula or verrucous lesion with distinct margins. Lesions can be white, pigmented or ulcerated (del Pino et al. 2013). The multifocality of the lesions also concerns the vagina, and the perianal and cervical area (Goffin et al. 2006). dVIN is typically a unifocal macula, a thickened lesion or erosion- sometimes a wound that does not heal. It can be itchy or sore as well as asymptomatic (del Pino et al. 2013). The treatment of precursor lesions is primarily surgical, but HSIL can also be treated with laser or imiquimod 5% cream (Westermann et al. 2013, van de Niuewenhof et al. 2009). Therapeutic HPV vaccination in the management of HSIL has also been studied, with promising results: 15 out of 19 studied HSIL patients had clinical response and complete response was achieved in 9 out of 19 HSIL patients (Kenter et al. 2009).

2.2.4 Protein markers of premalignant vulvar lesions

In dVIN, the immunostaining for p53 can show two different staining patterns: either positive nuclear or p53-null (separated from patchy p53 normal staining). In turn, p16 positivity is rarely detected in dVIN (Sighn et al. 2015, del Pino et al. 2013). HSIL is typically p16-positive, and weakly p53-positive (with 1-25% nuclei stain positive) (Sighn et al 2015, Hantschmann et al. 2005).

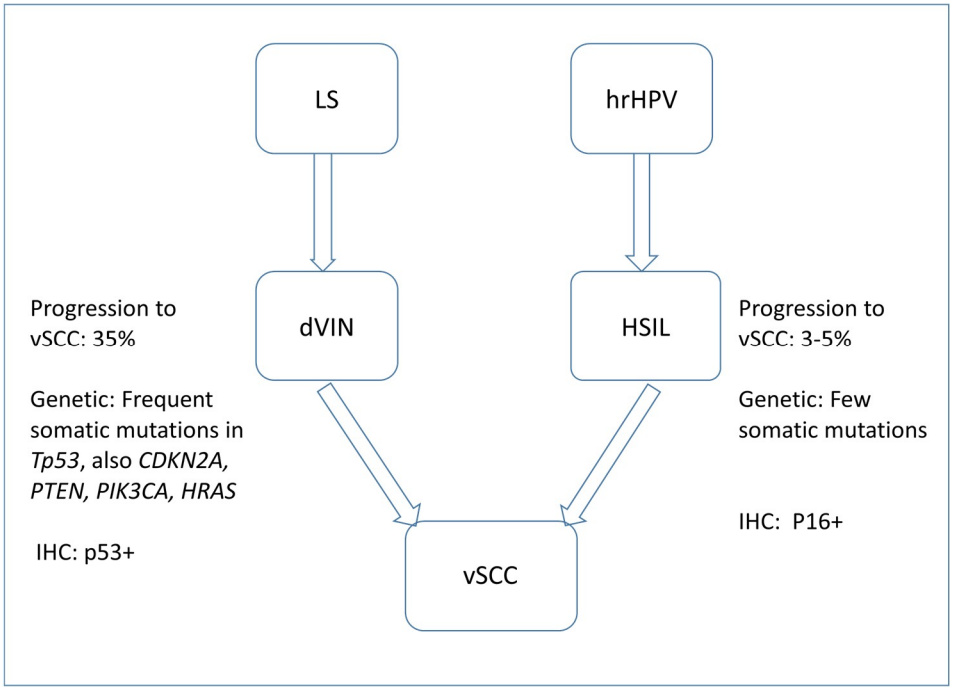

Figure 3. Two main pathways to vulvar SCC (modified from Singh et al. 2015, Trietsch et al. 2015).

2.3 Vulvar squamous cell carcinoma (vSCC)

2.3.1 Epidemiology and classification of vSCC

Vulvar cancer is a relatively rare gynecologic malignancy, representing roughly 4 % of the tumours of the female genital tract (Hacker et al. 2012). The annual incidence in Finland and in other developed countries is approximately 2 per 100 000 women (Trietsch et al. 2015, Finnish Cancer Registry 2017).

Squamous cell carcinoma represents 90% of the cancers of the vulva. Other types of cancer of the vulva are melanoma, verrucous carcinoma, Paget's disease, adenocarcinoma, basal cell carcinoma and Bartholin's gland carcinoma (Hacker et al. 2012). Traditionally, the classification of vSCC is based on surgical findings revised by the International Federation of Gynaecology and Obstetrics (FIGO), referred to as the FIGO staging, regarding tumour size, invasion, and lymph node or distant metastasis (Table 1). The current classification was established in 2009 (Hacker et al. 2012). The overall 5-year survival is strongly associated with the

number of lymph node metastasis, being approximately 72% for stage I, but only 34% for stage IIIC (van der Steen et al.2010).

Stage I	Tumour confined to the vulva
IA	Lesions < 2cm and stromal invasion <1mm
IB	Lesion >2cm or stromal invasion > 1mm
Stage II	Tumour invasion to adjacent perineal structures (1/3 lower urethra, 1/3 lower vagina, anus)
Stage III	Tumour with inguino-femoral lymph node metastases
IIIA	1 lymph node metastasis
IIIB	2 or more lymph node metastases
IIIC	>3 lymph node metastases with extracapsular spread
Stage IV	Tumour invades other regional (2/3 upper urethra, 2/3 upper vagina) or distant structures
IVA	Tumour invades regional structures
IVB	Tumour with distant metastases

Table 1. FIGO staging from 2009, table modified from the FIGO cancer report 2012 (Hacker et al. 2012).

2.3.2 Histopathology of vSCC

By histology vSCC is divided into three subtypes: the basaloid, warty and keratinizing types, the last one being the most common type and mainly associated with LS (del Pino et al. 2013). Basaloid tumors compose of keratinocytes that resemble basal epidermal cells with little keratinization. Warty types exhibit irregular nests, often with keratinization. Keratinizing vSCC shows an invasion of atypical epidermal keratinocytes into the dermis with large nuclei, and keratin pearls can also be seen (Figure 4). The overlap of these histological subtypes of vSCC does exist (del Pino et al. 2013). vSCC is classified into three grades in terms of differentation: I) well differentiated, II) moderately differentiated and III) poorly or undifferentiated, where well-differentiated vSCC shows minimal pleomorphism and abundant keratin pearls, and poorly differentiated tumors show a high level of nuclear atypia and a minimal amount of keratinization (Hacker et al. 2012).

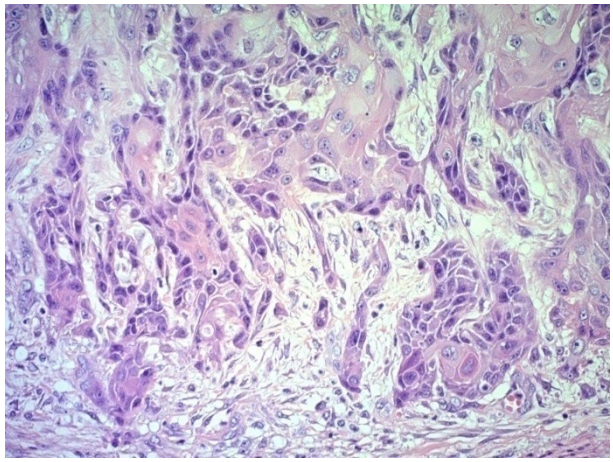

Figure 4. The histology of moderately differentiated keratinizing vSCC shows nests of atypical keratinocytes, with eosinophilic cytoplasm and atypical large nuclei. *HE staining, original magnification x200.* Histological picture by R.H-O.

2.3.3 Aetiology of vSCC

Vulvar SCC has two aetiological pathways: hrHPV-dependent and HPV-independent/LS-dependent pathways (Figure 3).

The atributable role of LS in vSCC varies between 9%-76% of the cases. The largest study from the US arrived at the prevalence of 36% in vSCC patients (Davick et al. 2017). Another main pathway is associated with HPV and is found in 15%-79% of vSCC cases depending on the geographic area, with an estimated 35%-50% of vSCC cases in the Western population (van de Niuewenhof et al. 2009, del Pino et al. 2013). HPV 16, 33 and 45 are high-risk types detected in vSCC and its precursor HSIL (Skapa et al. 2007). The HPV-dependent pathway associates with smoking, a higher number of sexual partners and immune suppression (Trietsch et al. 2015). It affects women at a relatively younger age (30-50 years) than LS-dependent vSCC, which develops in women of a more advanced age (60-80 years) (Hoang et al. 2016). These two aetiological routes differ not only in terms of premalignant lesions, but also the risk factors, histological subtypes and genetic alterations (Dong et al. 2015). Naturally, there is overlapping between these two pathways, and, for example, the same histological types (like keratinizing SCC) or molecular alterations (e.g. p53 immunopositivity) can exist in both pathways (del Pino et al. 2013). In general, the pathogenesis of the HPV-related pathway has been better defined than that of the LS-dependent pathway.

2.3.4 Genetic alterations in vSCC

Somatic mutations, allelic imbalance, loss of heterozygozity, copy number changes, microsatellite instability and epigenetic changes are genetic alterations detected in vSCC and its precursors (Trietsch et al. 2015). In HPV-related vSCC, somatic mutations are less likely to exist than in LS-dependent vSCC, but allelic imbalances occur equally in both. Studies on epigenetic changes in vSCC are few, and a comparison between the different pathways cannot be made. Taken together, HPV-independent vSCC harbours more somatic mutations and is associated with a worse prognosis than HPV-related vSCC without genetic or epigenetic changes (Trietsch et al. 2015).

Tp53 and other somatic mutations in vSCC

Tumour suppressor gene *Tp53* mutations are a frequent event in almost all malignant tumours (Sadalla et al. 2011). Of all mutations in vulvar SCC, *Tp53* mutations are the most often detected (Trietsch et al. 2015). Missense and deletion mutations of *Tp53* are an early event in the LS-dependent pathway, with reported frequencies as high as 70% for LS, 60% for premalignant vulvar lesions, and 81% for vulvar cancer cases. However, taken together all the studies in this field, remarkably lower frequencies are reported (6% for LS, 16% for VIN and 26% for vulvar carcinoma) (Dong et al. 2015, Trietsch et al. 2015).

Somatic mutations of *cyclin-dependent kinase inhibitor 2A (CDKN2A)* have not been detected in LS or dVIN/HSIL but occur in 0%-15% of HPV-negative vSCC cases (Trietsch et al. 2015). The *CDKN2A* gene codes the tumour suppressor proteins p16 and p14. Also $p14^{ARF}$ mutations can be detected in some vSCC cases (14%) and are considered as marker of poor prognosis in vSCC (Lavarato-Rocha et al. 2013). In addition, mutations in other genes such as *phosphatase and tensin homolog gene* (*PTEN*, 0%-60% of cases), *epidermal growth factor receptor* (*EGFR*, 12% of vSCC), *harvey rat sarcoma viral oncogene homologue* (*HRAS*, 11% of vSCC) and *phosphatidylinositol-4,5-bisphosphate 3-kinase* (*PIK3CA,* 8% of vSCC), have been detected, but their prognostic value is unknown (Trietsch et al. 2015, Palisoul et al. 2017).

A decreased expression of *interferon regulatory factor 6 (IRF6)*, a tumour suppressor acting in keratinocytes, is detected in the carcinoma process from LS to vSCC. *IRF6* promoter hypermethylation is considered to be an early event in the development of vSCC from LS (Rotondo et al 2016). Furthermore, the hypermethylation of the genes *O-6-methylguanine-DNA methyltransferase (MGMT)* and *RAS-association domain family 2 (RASSF2)*, which function in protecting the genome and regulating the cell cycle and apoptosis, are associated with vSCC (Guerrero-Setas et a. 2013). The *RAS homologue gene family A (RhoA)* has many tumour promoting effects, and its overexpression is associated with vSCC (Wang et al 2016). *Rho-associated coiled-coil-containing protein kinase 1 (ROCK1)* is a downstream effector of *RhoA*, and its expression is decreased in vulvar SCC when compared with adjacent normal vulvar epithelium. Lower expression levels of *ROCK1* are correlated with worse survival rates and poor prognosis (Akagi et al. 2014).

2.3.5 p53 and p16 immunohistochemistry in vSCC

Tp53 mutations lead to a more stable p53 protein and its accumulation to nuclei, which can be detected by immunohistochemistry. The p53-null phenotype can also be detected, which is due to nonsense or deletion mutations in *Tp53* gene. Some recent studies have implicated p53 immunopositivity as a marker of a poor prognosis of vSCC, but others have found no difference in survival (del Pino et al 2013, Dong et al. 2015, Hay et al. 2016). HPV-dependent vSCC is, in most cases, negative for p53 immunohistochemistry, but, in minority of cases, p16 and p53 coexist (Hay et al. 2016).

Infection of cells with hrHPV types lead to oncoprotein E6 and E7 production, which, in turn, promotes tumour progression. E6 binds to p53 leading to its degradation, and E7 mainly affects the retinoblastoma protein (pRb)-pathway but also several other cell cycle regulators and cell proliferation factors (Mangino et al. 2016). Since pRB is a negative regulator of p16, the inactivation of pRb by HPV leads to the upregulation of p16. This, in turn, can be detected by means of immunohistochemistry (Hay et al. 2016). Therefore, p16 immunopositivity can be used as a marker of tumor HPV (Cheng et al. 2016).

2.4 Oestrogen related receptors (ERRs)

Oestrogen-related receptors (ERRs), including ERRα, ERRβ and ERRγ, are a family of nuclear transcription factors (American nomenclature committee NR3B1, NR3B2, and NR3B3, respectively) (Tremblay et al. 2007). ERRs participate in the oestrogen signaling pathway through oestrogen receptors (ERs), but their most important physiological role involves the regulation of numerous genes involved in cell metabolisms, the cell cycle and mitochondrial respiration (Ariazi et al. 2006, Giguere 2008, Eichner et al. 2011). Unlike ERs, ERRs do not bind oestrogen itself, and are thus called orphan receptors (Tremblay et al. 2007). ERRα can be considered a master regulator of metabolism: it regulates genes involved in lipid uptake, mitochondrial biogenesis, fatty acid oxidation and glucose metabolism, especially in tissues with high energy demands (Ariazi et al. 2006). ERRγ targets essentially the same genes as ERRα, but with either a suppressing or a promoting effect (Eichner et al. 2011). ERRβ is necessary for, for example, placental formation and nervous system development, but also plays a role in the stress response (Tremblay et al. 2007, Buerly 2013). ERRs have been studied in various cancer types, and ERRα overexpression has been discovered to be a marker of poor prognosis in breast, ovarian and colon cancer (Bianco et al. 2012, Bernatchez et al. 2013, Heckler et al. 2015, Kim et al. 2016). ERRs also play a role in immunological processes involving T-cell differentiation and macrophage activation (Sonoda et al. 2007, Mikhalek et al. 2011).

2.5 Serpin A1

Serpin peptidase inhibitor clade A member 1 (Serpin A1), also called alfa-1-antitrypsin (AAT), is a member of serine protease inhibitors (serpins). Serpins are divided into intracellular molecules (clade A) and extracellular molecules (clade B). The most important physiological function of Serpin A1 is the inhibition of neutrophil elastase activity in the lungs, and the heritable deficiency of Serpin A1 is associated with early-onset emphysema (Ehlers 2014). It also inhibits the activity of plasmin, thrombin, trypsin, chymotrypsin and plasminogen activator (Farshchian et al. 2011). Serpin A1 is an acute-phase protein synthesized by the liver, but many types of tumour cells also produce it (Kwon et al. 2014). Serpin A1 has a role in blood coagulation, angiogenesis and the remodeling of the extracellular matrix (Nissinen et al. 2016). Serpin A1 has been studied in various

cancer types and found to predict poor prognosis in gastric, lung, ovarian, cervical and colorectal cancers, as well as cutaneous SCC (Higashiyama et al. 1992, Kloth et al. 2008, Normandin et al. 2010, Farshchian et al. 2011, Kwon et al. 2014, Kwon et al. 2015). Serum concentrations of Serpin A1 are also elevated in oesophageal SCC, as well as gastric, prostate, lung and colorectal cancer (El-Akawi et al. 2008, Yang et al. 2010, Topic et al. 2011, Perez-Holanda et al. 2014, Zhao et al. 2015).

3 AIMS OF THE STUDY

Childhood lichen sclerosus is a rare and poorly studied entity with little data on the comorbidities, prognosis and the influence on the QoL. No tools are available for clinicians for the evaluation of the malignant potential of LS in each patient. The pathogenesis of LS-dependent vulvar SCC is mostly unknown. The aim of the study was:

1. To define the prevalence, referral indications, family history, clinical picture, comorbidities, treatments, complications, and course of childhood-onset lichen sclerosus, and to evaluate the effect of lichen sclerosus on the QoL.

2. To characterize the role of Oestrogen related receptors (ERRs) in healthy vulvar skin, lichen sclerosus, premalignant vulvar lesions and vulvar SCC and to analyse their value as progression markers in lichen sclerosus.

3. To examine the role of Serpin A1 (alfa-1- antitrypsin) in lichen sclerosus, premalignant vulvar lesions and vulvar SCC, and to evaluate its value as a progression marker, in addition to comparing Serpin A1 to tumour markers p53 and p16.

4 MATERIALS AND METHODS

The Ethics Committee of Tampere University Hospital approved and the Finnish National Supervisory Authority for Welfare and Health gave its official permission for the use of diagnostic histological LS and vulvar SCC samples from adults and minors in the study. The study was performed in accordance with the Declaration of Helsinki. Participation in the studies was voluntary and each patient providing a serum sample or control samples of healthy vulvar skin gave their written informed consent. The patient register files were accessed with the permission of the Chief Medical Director of the Tampere University Hospital.

4.1 Patients and samples (I, II, III)

The register study (study I) included childhood-onset LS patients who were treated at Tampere University Hospital during 1982-2011. Patients were found from the patient record files of Pirkanmaa Hospital District by the using following criteria: ICD-10 code L90 or L90.0 (or, before year 1987, 7010B, 6981A), and age under 19 years. The data were collected by Dr. Kaisa Karvinen as a part of her master's degree thesis using a structured protocol. The register study included 46 childhood-onset LS patients. The study focused on girls only, and two boys were excluded.

A questionnaire (study I) along with an invitation to attend to clinical examination visit was sent to the childhood-onset LS patients who were found in the register study.

Study II included 128 and study III 74 LS and vulvar SCC patients from the Department of Gynaecology and Obstetrics or the Department of Dermatology at Tampere University Hospital during 1981-2014. Study II consisted of a total of 203 and study III of 120 diagnostic biopsy samples or samples obtained from vulvectomies (Table 2). Healthy vulvar skin samples were collected from volunteer study patients in 2007 by Dr. Reita Nyberg. All vSCC patients were staged according to the FIGO 2009 staging.

	ERRs		Serpin A1	
	Patients	Samples	Patients	Samples
LS, minors	24	24	0	0
LS, adults	51	51 LS 17 NVS	30 LS	30 LS
vSCC, LS-dependent	33	33 vSCC 5dVIN 32 LS 11 NVS	27	24 vSCC 3 dVIN+ 2HSIL 23 LS 9 NVS
vSCC, LS-independent	17	17 vSCC 1 HSIL 9 NVS	17	16 vSCC 4 HSIL 9 NVS
Extra-genital LS, no vulvar manifestations	3	3	0	0
	128	203	74	120

Table 2. Samples and patients in IHC studies (studies II and III). NVS= normal vulvar skin, LS= lichen sclerosus, dVIN= diffentiated vulvar intraepithelial neoplasia, HSIL= high-grade squamous intraepithelial lesion, vSCC= vulvar squamous cell carcinoma, ERRs= Oestrogen related receptors

Study III included serum samples collected from 84 volunteers by Dr. Reita Nyberg: 29 patients with vSCC before surgical treatment, 15 surgically treated vSCC patients during follow-up (on average 2.6 years after surgery), and 30 LS patients without malignant progression. As negative controls 10 age-matched uterine prolapse patients volunteered. Patients with a history of other tumors, acute infection or other inflammatory diseases were excluded.

4.2 Methods

4.2.1 Register and questionnaire study (I)

The register study focused on the predisposing factors, clinical phenotype, symptoms, histopathology, therapy, outcome, comorbidities and family history of LS.

With a questionnaire, further data was collected concerning the family history of LS, smoking, gynaecological and autoimmune disease comorbidities, current LS symptoms (iching, pain, bleeding, constipation, dysuria), the treatments and surveillance of LS, as well as infections (herpes, pinworms, condylomas or bacterial infections of the vulva) and possible history of sexual abuse. The impact of LS on the QoL was assessed with the question, "Has LS reduced your quality of life?" For those patients who did not reply, the questionnaire was re-sent. The questionnaire also included an item with which the respondents were askes for the permission to use previously taken diagnostic histological samples and to have the invitation to clinical examination visit sent to them.

The research visit to the clinic included a clinical examination by dermatologist Satu-Leena Laasanen and specialist in adolescent gynaecology Minna Joki-Erkkilä, in addition to the filling out of the DLQI and 15D questionnaires and the collection of blood samples for screening for other autoimmune diseases and infections. The data from the QoL questionnaires was analysed by the author. DLQI is a validated dermatology-specific questionnaire consisting of 10 questions, each scored from 0 to 3 points. The sum of the scores gives a result between 0 and 30 points, where 0-1 = no effect at all on the patient's life, 2-5 = small effect on the patient's life, 6-10 = moderate effect on the patient's life, 11-20 = very large effect on the patient's life and 21-30 = extremely large effect on the patient's life (Finlay et al. 1994, Basra et al. 2008). The 15D questionnaire is a generic, standardized instrument for assessing the health-related quality of life (HrQoL), which includes 15 questions with 5 options to choose from. The results are calculated by comparing population-based preference weights to the dimensions scoring between 0.0 (being dead) and 1.00 (no problems on any dimension) (Sintonen 2011). A difference 0.015 is the minimum important change in the 15D scores (Alanne et al. 2015).

All the available former diagnostic biopsy samples from childhood LS patients (26 samples) were re-read to confirm the diagnosis of LS by dermatopathologist Riitta Huotari-Orava (study I).

4.2.2 Immunohistochemistry (IHC) (II and III)

The immunohistohemical stainings were performed using a Ventana BenchMark immunostainer or automated immunostaining devise by Ventana Medical Systems SA, Illkirch, France and a Ventanan Ultraview DAB Detection Kit (Ventana, Tucson, Arizona) according to the manufacturer's instructions. Ultrablock antibody diluent (Ventana, cat.nro. 251-018) was used for p16 staining. The staining protocol for ERRs, p53 and p16 was planned by dermatopathologist Riitta Huotari-Orava, laboratory assistant Eini Eskola and the author, and the stainings were performed at the Pathology Laboratory, University of Tampere. Serpin A1 stainings were performed by Liisa Nissinen, PhD, at the University of Turku. All IHC stainings were evaluated by the author and the dermatopathologist (R.H-O) independently, and intraobserver and interobserver reproducibility was tested by re-evaluating part of the samples independently at different times.

Antigen	Product number	Source	Dilution
ERRα	H5844-00 (monoclonal)	Perseus Proteomics, Tokyo, Japan	1:100
ERRβ	PP-H6705-00 (monoclonal)	Perseus Proteomics, Tokyo, Japan	1:100
ERRγ	PP-H6812-00 (monoclonal)	Perseus Proteomics, Tokyo, Japan	1:25
Serpin A1	No A0012 (polyclonal)	Dako	1:7000
p53	Bp53-11 (monoclonal)	Ventana	prediluted
p16	E6H4 (monoclonal)	Ventana	prediluted

Table 3. Antibodies used in the immunohistochemical studies (Studies II and III).

Abdominal skin, liver tissue, high-grade serose adenocarcinoma and cervical carcinoma in situ were used as positive controls for ERRs, Serpin A1, p53 and p16, respectively.

In ERR study (study II) the intensity was scaled as follows: +++= stained as control/normal vulvar skin, ++= moderately decreased staining, += substantially decreased staining, basal epidermal layer stained slightly but upper epidermis unstained, 0= unstained. The positive control (abdominal skin) showed cytoplasmic staining for ERRα, nuclear staining for ERRβ, and both nuclear and cytoplasmic staining for ERRγ.

In the Serpin A1 study (study III) cytoplasmic staining was considered positive and the stainings scored as follows: negative (0), mild (+), moderate (++) and strong/as positive control (+++). The staining intensity was evaluated from the most invasive area of tumour.

The staining intensity of the p53 (study III) was scored <1% or null, 1%-10%, 10%-50% and >50% of tumour cell nuclei positive. For statistical analysis, more than 50% of tumour nuclei stained was considered positive in concordance with clinical practice and previous studies (Dong et al. 2015, Liegl et al. 2016). In LS, both continuous and discontinuous "band-like" basal epidermal keratinocyte staining was considered positive. Less than 10% nuclei positivity in LS was considered negative in concordance with normal vulvar skin where single epidermal keratinocytes and melanocytes stain.

The staining of p16 was interpreted as positive when over 75% of the cells showed intensive cytoplasmic and nuclear staining, so-called "block positivity". This "block positivity" associates strongly with HPV and can be used as a surrogate marker for high-risk HPV infection (Cheng et al. 2016).

4.2.3 Analysis of serum concentrations of Serpin A1 (III)

The serum samples were collected into Venosafe serum tubes, centrifuged at 2000 g for 10 minutes and stored in cryotubes at -70°C until use. An immunonephelometric assay (BM ProSpec automatic analyzer, Siemens Healthcare Diagnostics Inc., Siemens Aktiengesellschaft, Munich, Germany) was used for the analysis of serum concentrations of Serpin A1.

4.2.4 Statistical analysis

Clinical and survival data including the FIGO staging of patients and the histological grade of tumours were collected from patient records by the author. The mean follow-up time for vSCC patients was 3.9 years in study II and 8.1 years in study III. Pearsons' chi-square test was used in comparing the intensity of Serpin A1 between different patient groups. Fisher's exact test was used to determine the association between Serpin A1 staining and categorical variables (histological grade, FIGO staging, and immunopositivity of p53 and p16), and to determine the association between ERRα staining and architectural changes in the vulva. Kaplan-Meier analysis was used in survival analysis in studies II and III. The Kruskal-Wallis Test was used to assess the association between the duration of the LS symptoms and ERRα staining. Statistical analyses were performed by statistician Heini Huhtala from the University of Tampere.

5 RESULTS

5.1 Studies on childhood lichen sclerosus (I)

Study I composed of a register study, questionnaire study and clinical examination visits. The register study found 44 girls with childhood-onset LS. Fifteen (34%) of these childhood-onset LS patients completed the questionnaire, and 12 (27%) attended the clinical examination.

5.1.1 Register study on childhood lichen sclerosus

Description of our study population and referral indications

Our study found 44 girls with childhood-onset LS. The population of girls under 19 years of age in the Tampere University Hospital area is approximately 50 000, which yielded a prevalence of childhood-onset LS of 1:1100. The onset of symptoms occurred, on average, at 7.1 years of age (range 2-18 years). The mean diagnostic delay was 1.3 years (0-8 years). The mean follow-up period for the patients was 2.6 years (0-11.7 years) at the end of the register study. A diagnostic biopsy sample was available from 26 (59%) of the 44 patients.

LS was misdiagnosed in 37 (84%) of the 44 girls prior to the referral. The referral indications included non-specific inflammation of vulva in 10 (23%), dermatitis in 8 (18%), pruritus in 5 (11%), unpigmented vulvar skin lesions in 4 (9%), delayed puberty in 3 (7%) and genital bleeding in 2 (5%) girls. One girl was referred due to a suspicion of sexual abuse, and a suspicion of sexual abuse arose during the hospital visits for two other girls.

Symptoms and signs

The most common symptom of childhood-onset LS is vulvar itching, reported by 70% of our 44 patients. Other symptoms were dysuria (43%), pruritus in the anal area (16%), and constipation (11%). In total 16% of the patients were

asymptomatic. Lesions were located in the genital area in 40 girls (91%). Four (9%) had both genital and extragenital lesions. The extragenital lesions were multiple and the disease also remained active at the end of the register study follow-up period. Four girls (9%) had extragenital LS lesions only. A secondary anogenital infection was diagnosed in 15 patients (34%).

Management and complications of LS

Forty-two patients (95%) were treated with topical corticosteroids, and superpotent or ultrapotent topical corticosteroids were used in 38 patients (86%). Twenty-one patients (48%) were using mild potent corticosteroids as the only or an alternative treatment. Calcineurin inhibitors (tacrolimus and pimecrolimus) were used by 7 patients (16%), when the topical corticosteroid treatment had failed. Altogether, architectural changes (strictures and synechias) were detected in 8 patients -seven of these 8 patients (88%) had used topical superpotent or ultrapotent corticosteroids, and one had used topical moderate potent corticosteroid, and four (50%) had also used calcineurin inhibitors. Four (50%) of the patients with scarring used topical mild potent-corticosteroids occasionally as an alternative treatment for more potent corticosteroid ointments.

Comorbidity of LS

Six girls (14%) had an autoimmune comorbidity: morphea in two, vitiligo in two, granuloma annulare in one and celiac disease in one. Ten girls (23%) were atopic. Two girls (5%) had Turner's syndrome, and one of them had a horseshoe kidney. Two other girls had a kidney disease (a polycystic kidney disease and nephrotic syndrome). One had a delayed puberty of unknown aetiology.

5.1.2 Questionnaire study on childhood lichen sclerosus

Fifteen patients (34%) with childhood-onset LS completed the questionnaire. The questionnaire revealed that the recurrence of the symptoms is common: in 50% (3/6) of the patients who were asymptomatic at the end of the register study, a recurrence of symptoms had occurred. None of these patients were under surveillance. In total 44% (4/9) of the patients who had active LS at the time of the register study were still symptomatic. Reduced QoL was reported by 67% of the patients.

The register and questionnaire studies showed a positive family history of LS in four patients (9%). One patient reported LS in a first-degree relative (sister) and three in second-degree relatives (grandmothers).

5.1.3 Clinical examinations (unpublished data)

Overall, 12 patients with childhood-onset LS attended the clinical examinations in 2011 (5-29 years of age at the time of visit, median 12 years). Nine of the 12 patients (75%) were symptomatic at time of the examination; one patient had extragenital symptoms only. All patients completed the DLQI and 15D questionnaires. Four out of the 9 symptomatic patients (44%) reported a reduced QoL in DLQI, two patients reported LS having a moderate (6-10 points of max. 30 points) and two a small effect (2-5 points of max 30 points) on the QoL (mean score 5.0). All nine symptomatic patients reported a reduced QoL in the 15D questionnaire (0.8477-0.97, av. 0.94, on a scale of 0-1), which was associated with difficulties in urinating and defecating, symptoms (itching and pain), sleeping problems, or depression (Figure 5). One patient reported problems in the sexual life. Asymptomatic patients did not report reduced QoL.

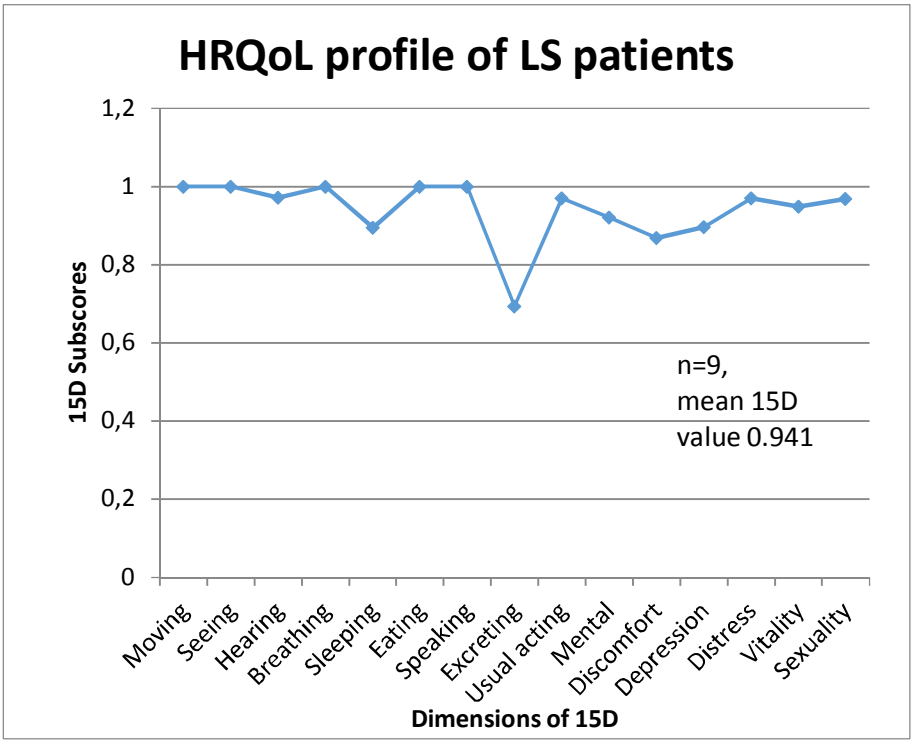

Figure 5. HRQoL profiles in 9 childhood-onset LS patients.

Blood samples were analysed for haemoglobin, leukocytes, CRP, rheumatoid factor, and thyroid function and the tests showed normal results in all patients. Antinuclear (ANA), extractable (ENA), thyroidea antibodies, as well as *Borrelia burgdorferi* antibodies were also tested, with negative results in all patients. Elevated IgE levels were detected in two, and fasting blood glucose levels were also elevated in two patients. Furthermore, *Chlamydia trachomatis* and *Neisseria gonorrhoaea* nucleic acid were controlled from urine samples and showed negative results in all patients.

5.2 Oestrogen related receptors in lichen sclerosus and vSCC (II)

Oestrogen related receptors were expressed in healthy vulvar skin samples (37 samples) as follows. ERRα was detected in the cytoplasm of the epidermal keratinocytes. ERRβ showed nuclear staining in epidermal keratinocytes and in the dermis, and macrophages/Langerhans cells were stained. ERRγ showed both cytoplasmic and nuclear staining in epidermal keratinocytes with the most intensive

stain in the basal layer and the sweat glands, while follicles and lymphocytes/macrophages stained in the dermis. The staining of normal vulvar skin was equal among all age groups and was independent of hormonal status. The control staining of the abdominal skin showed similar results. Therefore, the staining pattern of normal vulvar skin was used as a positive control and was scaled as +++ (Figure 6A).

ERRα decrease (+ or ++) was seen in 79% (19/24) of childhood-onset LS, in 51% (26/51) of adult-onset LS without malignant progression and in 59% (19/32) of LS with a malignant progression compared with the normal vulvar skin (Figure 6B). No correlations between the ERRα staining and the duration of LS symptoms or scarring of the vulva were seen ($p=0.367$ and $p=0.485$, respectively). In extragenital LS samples, the level of ERRα staining decreased in one (+) and stained similarly to the control in two samples (+++).

A substantial reduction or loss of ERRα cytoplasmic staining was detected in all 50 vSCC samples (graded 0 or +). In 8/33 (24%) LS-dependent and 10/17 (59%) LS-independent vSCC samples, there was a shift from cytoplasmic to nuclear ERRα staining (Figure 6C). Futhermore 4 out of 5 dVIN and one HSIL detected next to a carcinoma area showed a relative decrease in cytoplasmic ERRα staining compared to normal vulvar skin in 5/6 and compared to LS in 4/6 samples. Therefore, the ERRα staining intensity decreased from LS to dVIN/HSIL and futher to vSCC lesions (Figure 6D).

The shift from cytoplasmic to nuclear ERRα staining had no correlation to with patient survival and the FIGO staging of the patients. Nuclear staining may be associated with a higher histological grade; however, this finding was not statistically significant (nuclear staining for histological grade I, 27.8%; for grade II, 37% and for grade III, 40.0%; $p=0.665$).

The expression of ERRβ and ERRγ in the LS and SCC lesions did not differ from that of normal vulvar skin.

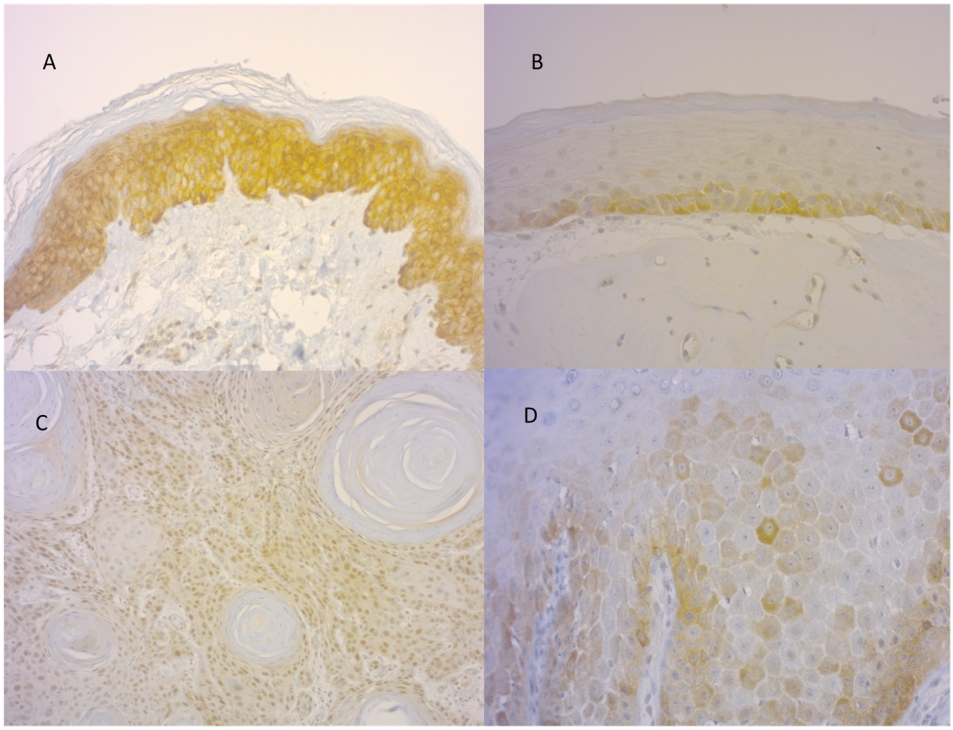

Figure 6. ERRα expression in A) cytoplasm of epidermal keratinocytes in healthy vulvar skin (scaled +++) B) lichen sclerosus (+) C) vulvar squamous cell carcinoma (vSCC), cytoplasmic staining shifted to nuclear staining, and D) differentiated vulvar intraepithelial lesion (dVIN) (++) and vSCC (+). The staining decreased gradually over the malignant process. *Original magnifications x200*. Reprinted from Gynegologic Oncology, 139, Lagerstedt M, Huotari-Orava R, Nyberg R, Mäenpää JU, Snellman E, Laasanen SL. Reduction in ERRα is associated with lichen sclerosus and vulvar squamous cell carcinoma, 535-540, Copyright (2015), with permission from Elsevier.

5.3 Serpin A1 in lichen sclerosus and vSCC (III)

5.3.1 Immunohistochemical studies of Serpin A1

The epidermal layer was negative for Serpin A1 staining in all 18 samples of normal vulvar skin (Figure 7A). Of all the 53 LS samples, 40% were negative, and 57% showed weak (graded +) or moderate (++) cytoplasmic Serpin A1 staining (Figure 7B). LS samples without malignant progression showed more intensive Serpin A1

staining than LS samples from vSCC patients (Figure 8). In the 9 HSIL/dVIN samples the staining was weak or moderate (Figure 7C) in 67% of the samples. The majority of the vSCC samples (30/40) showed tumour cell-specific strong (+++) (Figure 7D) or moderate cytoplasmic staining, and 13% (5/40) showed weak tumour cell-spesific Serpin A1 staining. Tumour cell-specific Serpin A1 was equal in LS-depenedent and LS-independent vSCC, and therefore independent of the aetiology. The expression of Serpin A1 was significantly higher in vSCC than in healthy vulvar skin, LS or HSIL/dVIN samples (Figure 8).

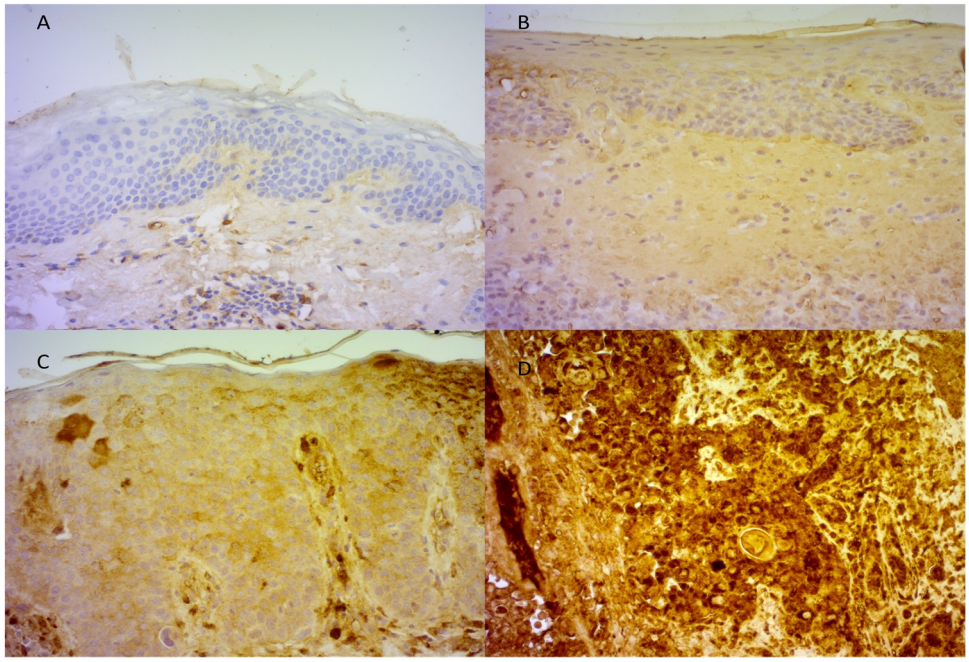

Figure 7. Serpin A1 expressions in healthy vulvar skin, lichen sclerosus, differentiated vulvar intraepithelial neoplasia (dVIN) and vulvar squamous cell carcinoma (vSCC). A) The epidermal layer of healthy vulvar skin is negative for Serpin A1. B) Weak (+) cytoplasmic staining of the epidermis in LS, and C) moderate intensity (++) staining of dVIN, and D) strong (+++) tumor cell-specific staining in vSCC. *Original magnifications x200. Histological photos by author and R.H-O.*

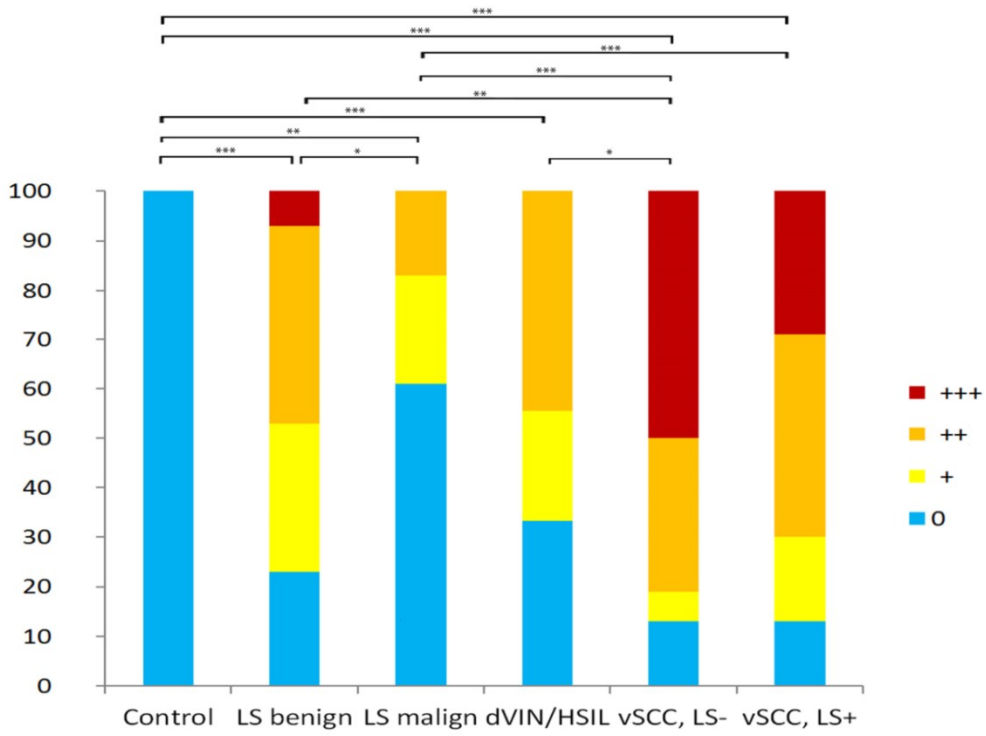

Figure 8. Semiquantitative analysis of Serpin A1 staining in healthy vulvar skin (n=18), lichen sclerosus (LS) without malignant transformation ie. benign LS (n=30), LS samples from vSCC patients ie. malign LS (n=23), differentiated vulvar intraepithelial neoplasia/high-grade squamous intraepithelial lesions (dVIN/HSIL) (n=9), LS-independent vulvar squamous cell carcinoma (vSCC) (n=16) and LS-dependent vSCC (n=24) samples. ***p<0.001, **p<0.01, *p<0,05 ($\chi 2$ test).

5.3.2 Serum analysis of Serpin A1

No statistically significant differences were detected in Serpin A1 serum concentrations between active vSCC, treated vSCC, LS or control groups. The mean values and range were 1.483 g/l (0.893-2.05g/l), 1.463 g/l (1.17-1.77g/l), 1.438g/l (0.95-1.78g/l) and 1.472g/l (1.15-2.01g/l), respectively. The normal range of Serpin A1 serum concentration is 0.96-1.78g/l.

5.4 Immunohistochemistry of p53 and p16 in lichen sclerosus and vSCC (III)

p53 was interpreted positive when over 50% of the tumour nuclei were intensively stained. Thus, 58% (14/24) of the LS-dependent and 38% (6/16) of the LS-independent vSCC samples were p53 positive (Figure 9A). Out of the LS-dependent and LS-independent vSCC samples, 17% (4/24) and 25% (4/16), respectively, were p53 null (<1% of nuclei stained) in comparison to normal vulvar skin (Figure 9B). Continuous or discontinuous "band-like" p53 positivity in basal epidermal cells was seen in 35% (8/23) of the LS samples from vSCC patients. However, LS without a malignant transformation was p53-positive in almost all of the cases (93%, 28/30). The difference of staining in LS was statistically significant ($p<0.001$) (Figure 9C).

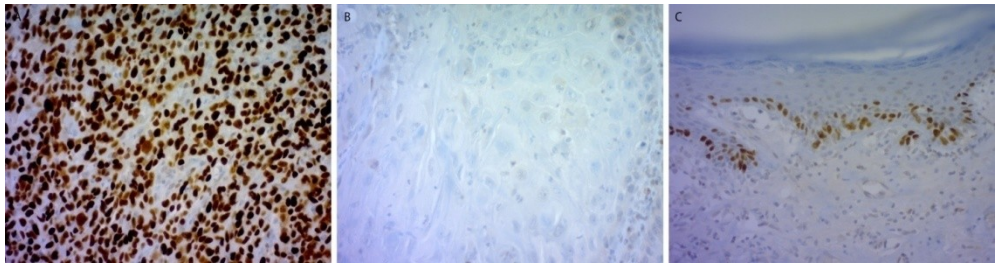

Figure 9. Expression of tumour marker p53 in lichen sclerosus (LS) and vulvar squamous cell carcinoma. A) p53-positive staining (over 50% of tumour nuclei stained) in vSCC, B) p53-null staining (<1% tumour nuclei stained) in vSCC and C) p53-positive "band-like" staining in the nuclei of basal epidermal keratinocytes in LS. *Original magnifications x200.* Histological photos by the author and R.H-O.

Positive p16 staining was detected in 13% (3/24) of the LS-dependent and in 50% (8/16) of the LS-independent vSCC samples (Figure 10A). A premalignant area next to a p16-positive carcinoma was also p16-positive in 3/4 cases (Figure 10B). All LS samples were interpreted as negative for p16, but mild/mosaic basal epidermal p16 staining was already apparent in LS samples next to p16 positive tumour (Figure 10C).

Positive staining for both p53 and p16 was detected in 3 (8%) vSCC samples.

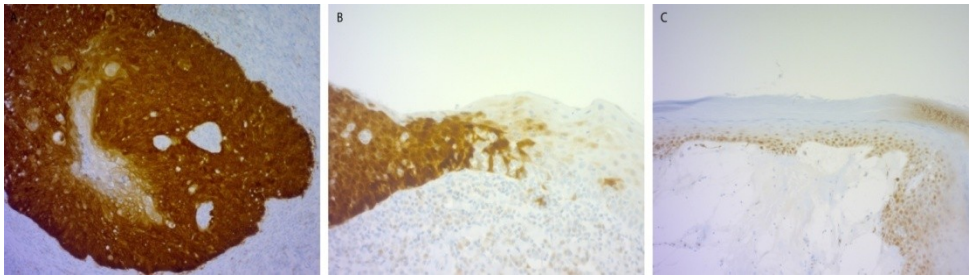

Figure 10. Expression of tumour marker p16 in lichen sclerosus (LS) and vulvar squamous cell carcinoma (vSCC). A) Intensive nuclear and cytoplasmic positivity of p16 in vSCC. B) p16 positivity in transition to HSIL, and C) mosaic/mild epidermal p16 staining in LS adjacent to a p16-positive vSCC, interpreted as p16 negative. *Original magnifications x200.* Histological photographs by the author and R.H-O.

There was a statistically significant correlation between p53 positivity and increasing Serpin A1 intensity (p=0.013*) in vSCC, but Serpin A1 and p16 positivity showed no correlation. No difference in overall survival was observed in LS-dependent or LS-independent vSCC patient groups (average follow-up time 8.1 years from diagnosis to the end of the study period, exitus in 63% (25/40) of the patients). The overall survival of vSCC patients showed no correlation with Serpin A1 intensity, nor with p53 or p16 positivity.

6 DISCUSSION

6.1 Childhood LS is a rare and challenging diagnosis

Due to its rarity and clinical variability, childhood-onset LS was misdiagnosed in 84% of the patients prior to referral. Our study yielded the prevalence of 1:1100 girls in hospital catchment area population, which is somewhat less than in a previous study on childhood LS prevalence (Powell et al. 2001). However, some milder or asymptomatic LS cases may be undiagnosed. The mean diagnostic delay of 1.3 years detected in our study is also in line with previous studies (Berthe-Jones et al. 1991, Ridley 1993, Powell et al. 2001, Jensen et al. 2012). This diagnostic delay can be very frustrating to the patients and their families. In addition, a false suspicion of sexual abuse, which occurred in three of our 44 register study patients, can be traumatic. All doctors treating pediatric patients should be aware of LS, and in the case of continuous itching and a burning sensation with whitish lesions in the genital area in a prepubertal girl should lead to a suspicion of LS. The training of general practioners and medical students regarding vulvar dermatosis should pay more attention to LS.

LS symptoms, vulvar itching and a burning sensation, as well as difficulties in urination and defecation, cause a reduced QoL in childhood LS patients. Fear and embarrassment are common in patients with vulvar dermatoses like LS (Lansdorp et al. 2013, van Cranenburg et al. 2017). In our questionnaire study, ten out of 15 patients (67%) reported a reduced QoL when asked "Has LS reduced your quality of life?" All patients who were symptomatic (9 out of 12 patients) at the clinical examination reported a reduced QoL in the 15D questionnaire, whereas the data from the DLQI questionnaire showed slightly or moderately impaired QoL in four (44%) of them (mean DLQI score 5.0). A previous study with 368 adult LS patients showed a very large impact on the QoL with mean DLQI score of 11.9 (van de Niuewenhof et al. 2010). It therefore seems that the DLQI questionnaire commonly used at dermatology clinics underestimates the burden of childhood-onset LS on the QoL. This warrants the development of an LS-specific QoL-instrument. In our study, four out of 12 childhood-onset LS patients (33%) reported difficulties in falling asleep, which may be due to a worsening of the

itching towards the evening (Fistarol et al. 2013). Previous studies on childhood LS have not reported an association with sleeping disorders, even though they are recognized in adult LS patients (Shasi et al. 2010). In conclusion, the 15D HrQoL-questionnaire seems promising in terms of measuring burden of the disease in childhood-LS patients, even though the questionnaire was originally developed for adult patients and for study purposes. Nevertheless, the development of an easily used LS-specific QoL instrument could introduce a better tool to clinical practice for the purpose of, for example, measuring the effect of treatments of LS patients. The association of sleeping disorders with childhood LS needs further studies. In addition, larger comparative QoL studies of childhood LS are needed.

The histology of LS is similar in all age groups, and the continuum of childhood-onset LS into adulthood could also indicate that childhood-onset and adult-onset LS are the same disease. Whether the same genes are involved and active in childhood- and adult-onset LS and the risk of complications is similar, is not known. Previous studies have found paediatric LS patients to have a positive family history of LS more often than adult LS patients (17% vs. 12%) (Powell et al. 2001, Fistarol et al. 2013). In our register study patients, a positive family history of LS was present in 4/44 (9%) patients. Familial LS can be partly explained by heritable HLA allele subtypes that make patients more susceptible to autoimmune diseases (Aslanian et al 2006). The familial form of LS may be associated with some germ-line mutations, which have not yet been defined. Notably, a positive family history of LS indicates that there are genetic alterations predisposing to LS and not to autoimmune diseases in general. This warrants the recruitment of LS families and further studies by, for example, exome sequencing. In our study, two familial paediatric LS patients (50%) also had another autoimmune disorder. A study by Aslanian et al. found a concomitant autoimmune disorder in up to 88% of familial LS patients (Aslanian et al. 2006). Whether familial LS patients are more susceptible to morbidity related to autoimmune disorders or have a more active disease, further highlights the importance of genetic studies focusing on LS families. Furthermore, genetic studies comparing the gene expression in childhood- and adult-onset disease could also bring more insight into the differences in associated morbidity and risk of complications.

In concordance with previous studies on childhood LS, 14% of our patients had autoimmune disease comorbidity: morphea in two, vitiligo in two, granuloma annulare in one and celiac disease in one girl (cf. Helm et al.1991, Powell et al. 2001, Oyama et al. 2003, Maronn et al. 2005). The prevalence of autoimmune diseases in childhood LS is high when compared to the 1% of prevalence of

autoimmune thyroid disease and celiac disease in the general population of Finnish children (Välimäki et al. 1999). These comorbidities should be excluded from all childhood-LS patients. Furthermore, in patients first diagnosed with morphea, an inspection of the genital area for LS should be performed. In a study by Kreuter et al., 1% of 89 paediatric and 5.7% of 328 adult morphea patients had concomitant LS (Kreuter et al. 2012). In another study of 76 adult morphea patients, genital LS was found in up to 38% of the patients. None of these 15 patients with symptomatic genital LS had spontaneously complained of the symptoms (Lutz et al. 2012).

Interestingly, there were two girls (5%) with Turner's syndrome in our study. A French study with 133 Turner patients found LS in 17% of the patients (Chacktoura et al. 2014). Some case reports have also been published about the association of Turner's syndrome and LS (Goolamali et al. 2012, Chacktoura et al. 2014, Haidapoulos et al. 2016). Whether this association is due to low estrogen levels or increased risk of autoimmune disease, is not known. Doctors treating Turner's syndrome patients should pay attention to symptoms and signs consistent with LS.

Scarring and changes in the vulvar architecture were observed in 18% (8/44) of the patients in our register study, even though 88% of these patients had used topical superpotent or ultrapotent corticosteroids. The frequency of disturbances in vulvar architecture is in line with earlier studies (Ellis et al. 2015). The EDF guideline recommends the use of ultrapotent or superpotent corticosteroid ointments daily for 1-3 months until the disease is in remission (Kirtchig et al. 2015). The remission of LS means the relief of LS symptoms with no evidence of epithelial abnormality or white hyperkeratosis, and no progression of scarring or adhesions (Ellis et al. 2015). Maintenance therapy with an individually planned regimen (from 2-3 times a week to 2-3 times a month) with moderate to superpotent corticosteroid ointment is recommended. The maintenance therapy and surveillance should be continued even up to several years (Ellis et al. 2015). For the development of scarring, there may be at least three risk factors. Firstly, dropping out of surveillance was common among our study patients, and 60 % of patients completing the questionnaire study were not followed over time. The previous study by Ellis et al. showed that childhood LS patients without surveillance developed scarring, a progression of the disease and infectious complications significantly more often than patients in surveillance. Notably, if LS was treated only according to the subjective relief of symptoms, scarring developed in 41% of patients (Ellis et al. 2015). Secondly, 48% of our patients used mild

potency corticosteroids either as the only initial treatment or in addition to more potent corticosteroids. The treatment of childhood LS with hydrocortisone 1% may relieve the symptoms without clinical recurrence (Ellis et al. 2015). Thirdly, maintenance therapy was not used by the majority of our patients. Our study patients were treated during 1981-2011 and the concept of maintenance therapy in LS was established less than 10 years ago: the British Association of Dermatology guidelines for LS from 2010 did not recommend maintenance therapy, while the EDF guideline from 2015 concludes that proactive maintenance therapy is often beneficial to female patients (Neill et al. 2010, Kirtchig et al. 2015). All these aspects may explain the high number of architectural changes in our patients.

There was no progression to vSCC in our childhood LS patients. There is one case report of a childhood-onset LS patient who developed vSCC at the age of 32 (Smith et al. 2009). However, the knowledge of risk factors for malignancy is limited (van de Niuewenhof et al. 2009, Lee et al. 2015). A progression from LS to vSCC seems to be independent of the follow-up time for patients (Halonen et al. 2017). Moreover, no common clinical features in 507 LS patients developing vSCC were identified in a prospective cohort study (Lee et al. 2015). There is, however, increasing evidence that adequate treatment and follow-up of LS patients decreases the risk of malignant progression (Cooper et al. 2004, Renaud-Vilmer et al. 2004, Lee et al. 2015).

The prevention of complications is essential in the management of childhood LS − the initial treatment should be effective enough to achieve remission, and the maintenance therapy should last years after remission. The follow-up should preferably take place in specialist unit to ensure a good QoL and normal vulvar architecture of childhood LS patients.

6.2 Biomarkers in the pathogenesis of LS and vSCC

Studies on biomarkers in LS have increased our understanding of the pathogenesis of LS, but none of the biomarkers have established a footing in clinical practice as a prognostic marker (Carlson et al. 2013). The most widely studied biomarker in LS and vSCC is p53, tumor marker involved in almost all cancer types (Sadalla et al. 2011). A commonly used marker of HPV in gynaecologic cancers, p16, is another biomarker in vSCC (Hoang et al. 2016). Our study brought out two new biomarkers, ERRα and Serpin A1, in the pathogenesis of LS and vSCC.

6.2.1 ERRα decrease associates with lichen sclerosus and vulvar SCC

To the best of our knowledge, this was the first study showing ERRα, ERRβ and ERRγ expressions in normal vulvar skin (Figure 5A). Even though the incidence of LS has two peaks at times of low oestrogen production, the role of oestrogen or oestrogen receptors in the pathogenesis of LS has not been established (Taylor et al. 2008, Powell et.al. 1999). As ERRs participate in oestrogen signalling, they may have been the missing link between oestrogen and LS, but the expressions of ERRα, ERRβ and ERRγ in the vulva were independent of age and hormonal status (Ariazi et al. 2006). ERRα expression has been shown to decrease in the vagina after menopause (Cavallini et al. 2008). However, vaginal and vulvar tissues differ from each other by their histological basis and, for instance, the steroid receptor ratios, since the vagina and vulva derive from different embryological origins (Puppo 2011). Concerning LS, the vagina is not involved (Powell et al. 1999).

Overall, 58% of the LS samples showed decrease in ERRα staining compared to normal vulvar skin (Figure 5B). This decrease was independent of the progression of LS and probably associates with inflammation driven by LS with two mechanisms. Firstly, ERRα is involved in the regulation of immunology and autoimmunity by participating in the metabolism of T cells and macrophages (Sonoda et al. 2007, Mikhalek et al. 2011, Yuk et al. 2015). ERRα reduction has previously been detected in one other autoimmune disease, rheumatoid arthritis (Bonnelye et al. 2008). The second mechanism could be related to oxidative stress and reactive oxidative species (ROS) induced by LS (Sandler et al 2004). ERRα, as a master regulator of energy metabolism, is an important factor regulating ROS production, a by-product of mitochondrial respiration (Giguere 2008). The production of oxidative reactive substances and the oxidation of lipids, proteins and DNA in LS may contribute to autoimmunity, to an increased risk of developing secondary malignancies, and to mechanisms causing scarring (Sander et al. 2004). Therefore, the use of antioxidants may show some benefit in LS patients, and EDF guidelines also notes vitamins A and E as potentially beneficial (Kirtchig et al. 2015).

Our study showed that ERRα reduction associates with a progression from LS to vSCC. A substantial decrease in or loss of ERRα expression was detected in all of our vSCC samples (Figure 5D). Interestingly, ERRα is up-regulated in many other cancer types, such as breast, ovarian, endometrial, colon, prostate, adrenocortical and thyroid cancers (Cavallini et al. 2005, Fujimoto et al. 2007, Fujimura et al. 2007, Fujimoto et al. 2009, Jarzabec et al. 2009, Deplois et al. 2013,

Felizona et al. 2013, Mirebeau-Prunier et al. 2013), but also in oral SCC (Tiwari et al. 2014). In concordance with our results, in another inflammation-driven cancer, hepatocellular carcinoma, the knock-out of ERRα promoted tumour growth (Hong et al. 2013).

Knowing the function of ERRα as a global regulator of energy metabolism, the reduction of ERRα in vSCC may favour tumour prognosis through impaired ROS levels, as discussed earlier. Several other pathogenetic mechanisms associated with ERRα reduction can also be proposed. In hepatocytes, ERRα knock-out and impaired ROS levels increased cell death due to necrosis instead of apoptosis, which has higher energy demands. Cell necrosis, in turn, induces tumour growth due to compensatory cell proliferation (Hong et al. 2013). Impaired ROS levels have an effect on many key cytokines and tumour suppressor genes associated with vSCC, such as p53, p21 and p16 (Gupta et al. 2012). ERRα also has a direct effect on NF-ϰB pathway activation, a pathway associated with various inflammation-related diseases and inflammation-associated cancers (Hong et al. 2013). In addition, as ERRα is knocked-out, the Wardenburg effect may take place in tumour cells. This means a shift in the metabolism to favor anaerobic glycolysis rather than the aerobic Krebs cycle. Cancer cells rely on glycolysis, especially in an inflammatory microenvironment (Yamacotghi et al. 2014).

The localization of steroid receptor expression may also play a role in cancer progression. A previous study has found oestrogen receptor β (ERβ) expression turning from nuclear to cytoplasmic in the progression of vulvar SCC and indicating a poor prognosis of these patients (Zannoni et al. 2011). In our study, a shift from nuclear instead of cytoplasmic ERRα staining was observed in 36% (18/50) vSCC samples (Figure 5C). The change in ERRα from a cytoplasmic to nuclear localization indicates a loss of protein function. Nuclear staining was not associated with prognosis or FIGO staging in our patients, but it seemed to increase in cancer progression in terms of histological grade. The limited number of patients and relatively short follow-up time (mean 3.9 years) are prone to reduce the statistical power of the findings.

The important role of ERRα in metabolism and its overexpression in many cancer types has prompted the development of ERRα inhibitors in the treatment of, for example, obesity and diabetes mellitus (Bianco et al. 2012, Hong et al 2013). Our findings suggest caution with these therapeutic applications, since blocking ERRα activity may promote inflammation-induced cancers such as vSCC.

In conclusion, the reduction of ERRα staining associates with the pathogenesis of LS and vSCC and confirms the hypothesis that vSCC is an inflammation-induced cancer. Unfortunately, ERRα is not useful as a prognostic marker in LS.

6.2.2 Serpin A1 overexpression in lichen sclerosus and vSCC

This was the first study showing that Serpin A1 was associated with LS and vSCC. Of all LS samples, 60% showed increased staining compared to healthy vulvar skin (Figures 6A, 6B and 7). Interestingly, LS in the absence of vSCC showed more intensive Serpin A1 staining than LS samples from vSCC patients. This suggests that Serpin A1 has cytoprotective and immunomodulating properties. These have been detected in previous studies on murine models of other autoimmune diseases, e.g. rheumatoid arthritis and type 1 diabetes (Ehlers et al. 2014).

Serpin A1 showed tumour cell-specific Serpin A1 overexpression in the majority (88%) of the vSCC samples, independent of aetiology. Serpin A1 also has many tumour promoting effects, such as the prevention of cell apopotosis and enhancement of tumour cell migration and invasion capacity (Normandin et al. 2010, Chan et al. 2015, Kwon et al. 2015). Previous studies have found that Serpin A1 is a marker of poor prognosis in gastric cancer, lung and colorectal adenocarcinomas, oesophageal and cutaneous SCCs, papillary thyroid carcinomas, anaplastic large cell lymphomas, and epithelial ovarian and HLA-positive cervical carcinomas (Higashiyama et al. 1992, Kloth et al. 2008, Normandin et al. 2010, Farshchian et al. 2011, Vierlinger et al. 2011, Kwon et al. 2014, Kwon et al. 2015). Our study did not find a correlation between Serpin A1 staining intensity and the overall survival, nor the histological grade or FIGO staging of our vSCC patients. Notably, the number of samples in our study was limited.

Serum concentrations of Serpin A1 did not differ between the controls, LS patients, patients with active vSCC, and surgically treated vSCC patients. Previous studies have found elevated plasma levels of Serpin A1 in oesophageal SCC, as well as gastric, prostate, lung and colorectal cancer (El-Akawi et al. 2008, Yang et al. 2010, Topic et al. 2011, Perez-Holanda et al. 2014, Zhao et al. 2015). Due to its physiological function as an acute-phase protein, Serpin A1 concentration in serum is influenced by various inflammatory processes, and it may thus not be sensitive enough for the evaluation of a malignant progression of vSCC (Ehlers et al. 2014).

Serpin A1 overexpression can be associated with LS and vSCC by several pathogenetic mechanisms. Firstly, Serpin A1 expression is enhanced by the

cytokines EGF, TNF-α, INF-γ and IL-1β in cutaneous SCC cells, and the latter three cytokines are also upregulated in LS (Farshchian et al. 2011, Terlou et al. 2012). Secondly, Serpin A1 interacts with the TNFα- NF-κB- axis, which is also associated with both HPV-infection and LS, and could therefore promote the progression to vSCC via both inflammatory pathways to vSCC (Bodelon et al. 2014, Ehlers et al. 2014, Hardikar et al. 2015). Also, ERRα participates in the NF-κB- pathway (Hong et al 2013). The NF-κB pathway is active in many inflammation-induced cancer types, especially in solid tumours, and the suppression of the NF-κB pathway, in turn, inhibits the proliferation and invasion of tumours (Aggarwal et al. 2009). Therefore, the NF-κB pathway may also play an important role in the pathogenesis of vulvar SCC. New therapeutic approaches targeting this pathway should be studied in vSCC.

Even though there is an increasing understanding of the role of chronic inflammation and tumour microenvironment in cancer progression, the studies in LS-dependent vSCC microenviroment are limited (Rutkowski et al. 2010, Nissinen et al. 2016). Matrix metalloproteinases 2 and 9 seem to have a role in cancer progression (Määttä et al. 2010). Unfortunately, therapeutic applications targeting matrix metalloproteinases have failed (Nissinen et al. 2016). Our study brought out a novel factor in the vSCC microenvironment, Serpin A1, with a role in regulating immunity, complement activation and remodelling the extracellular matrix (Silverman et al. 2001). More studies on the microenvironment of vSCC are warranted to provide new immunomodulatory cancer therapies in vSCC.

Serpin A1 overexpression correlated with p53 immunopositivity in vSCC, but showed no correlation with p16 positivity. There is one recent study on lung cancer cells which found an association with p53 and Serpin A1. Mutant p53 up-regulated Serpin A1 expression and promoted tumour invasion (Shakya et al. 2017). In conclusion, Serpin A1 overexpression associates with LS and vSCC being significantly higher in tumour cells than in normal vulvar skin or LS. Serpin A1 immunohistochemistry or serum concentrations seem not useful in predicting the prognosis of LS or vSCC. A better understanding of the tumour microenvironment of vSCC may provide new aspects for future studies on therapeutic applications.

6.2.3 p53 and p16 immunohistochemistry in lichen sclerosus and vSCC

Even though p53 is a marker of poor outcome in various cancer types, in LS it is rather a marker of ischemic stress than one of malignant potential (Sadalla et al. 2011). Our study confirms this hypothesis, since LS with malignant progression showed p53 positivity in 35% of samples, while LS without malignant progression was significantly more often p53 positive, showing positive staining in 93% of the samples. In total 70% (28/40) of the vSCC samples was either p53-positive or p53-null which indicates the essential role of *Tp53* mutations in vSCC.

In our study, p16 positivity was not detected in LS samples. Previous studies have indicated that the two etiological pathways are not always distinct. Concomitant LS and HPV infection were detected in 8% of female LS patients (Hald et al. 2017). Interestingly, LS and p16 positivity in vSCC samples coexisted in 3 (8%) of our 40 vSCC patients. It can thus be speculated that having two risk factors, hrHPV and LS, increase the risk of vSCC. This result suggests that HPV status should be determined in all LS patients and considered when planning the follow-up.

In the primary prevention of HPV-dependent vulvar SCC, vaccination for HPV is effective and targets hrHPV types detected in vulvar SCC (Joura et al. 2007, Lewis et al. 2016). Also, in the management of HSIL, HPV vaccination has also been studied with promising results (Kenter et al. 2009). Therefore, HPV vaccination is promising in the prevention of HPV-related vSCC.

The overall survival of our vSCC patients did not correlate with p16 or p53 positivity, even though previous studies have suggested that p16 is a marker of favourable prognosis in vSCC (Hay et al. 2016). However, the number of patients in study III was considerably low for survival analysis.

6.3 Study limitations

Due to the rarity of childhood LS, the study population was limited. Biopsy samples were only available from 26 of the 44 childhood LS patients, since the diagnosis of LS is increasingly made based on clinical findings in children. Biopsy can also be traumatizing for prepubertal girls. This also restricted the sample size of childhood LS in study II. However, the sample size was large enough to make a comparison of ERRα staining in LS samples from adults, and the results can thus be considered satisfactory. Of the 44 childhood LS patients identified in the

register study, only 15 girls (34%) completed the questionnaire and 12 (27%) attended clinical examinations. The difficulties in the recruitment of patients may be due to embarrassment related to symptoms involving the intimate parts or a fear of the pain the clinical examination may cause.

The sample size in the immunohistochemical studies was also limited, which limited the statistical power of our findings. However, our immunohistochemical studies, although preliminary, introduced two novel biomarkers to the pathogenesis of LS and SCC. Future studies with larger sample sizes are needed to emphasize their correlation with the prognosis of vSCC patients.

6.4 Future perspectives and clinical implications

Childhood LS is often misdiagnosed and diagnosis is delayed. Awareness of childhood LS should be enhanced among health care professionals seeing pediatric patents. The burden of the disease causes a remarkable effect on the QoL of the patients. However, the DLQI questionnaire used in clinical practice is not specific for LS and underestimates the effects on LS on the QoL. Larger studies on the QoL of paediatric LS patients are warranted, even though the 15D questionnaire seemed a promising tool in our study. Of the childhood-onset LS patients attending clinical examinations 33% reported sleeping disorders, which cannot be ignored. Whether all LS cases are genetically identical and whether there is a germ-line mutation predisposing to LS, should be further studied by means of genome-wide expression profiling or exome sequencing. The recruitment of familiar LS patients for these further studies is important.

The course of LS is unpredictable. Childhood LS does not resolve in puberty and relapses are common. The patients who dropped out from surveillance are in increased risk for complications. An effective initial treatment, maintenance therapy and long-enough surveillance of childhood LS patients are essential in preventing symptoms reducing the QoL and to ensure normal vulvar architecture throughout life. The focus of the follow-up of paediatric (and adult) LS patients should probably be more upon clinical findings on examination, not only on symptom control to prevent the complications. The risk of malignancy in childhood-onset LS is still unknown and needs further studies.

There are no biomarkers for clinical practice to predict the clinical outcome of LS. Our study confirms that tumour marker p53 immunopositivity does not predict the malignant potential. Positivity for p16 was never detected in LS, but in

three of our vSCC patients, LS and p16 positivity coexisted. Since positivity for p16 is a surrogate marker of hrHPV, this finding may indicate a higher risk for malignancy for these patients with two risk factors. The HPV status of LS patients should therefore be tested, and this should be considered during the follow-up of these patients. In the future, HPV vaccines are safe and effective in the prevention of HPV-related vSCC.

A decrease in ERRα and increase in Serpin A1 expression were detected in LS samples, with no correlation to LS progression, and their relevance as prognostic markers in LS is thus questionable. However, both biomarkers are related to vSCC development in both the LS-dependent and LS-independent pathways. A tumour cell-specific overexpression of Serpin A1 and decrease in ERRα in vSCC suggest that chronic inflammation is a major risk factor for vSCC, since both biomarkers have been previously linked to autoimmunity, inflammation and other cancers. Both markers are also associated to the NF-κB pathway providing an interesting field of research in the future. Also, better understanding of the microenvironment of vSCC would help to achieve a better understanding of the pathogenesis of vSCC, and maybe even provide new treatment modalities.

7 SUMMARY AND CONCLUSION

The current work was designed to explore the prevalence, clinical picture, comorbidities and prognosis of childhood lichen sclerosus and to identify immunohistochemical markers in LS and vSCC. The results of this study can be summarized as follows:

1. Childhood-onset LS is rare and often misdiagnosed. LS has a significant effect on the QoL of the patients, and the 15D questionnaire seems a promising tool for measuring the QoL in childhood-onset LS. The high risk for other autoimmune diseases should be kept in mind during the follow-up. To prevent the complications, the initial therapy should be effective enough to cure clinical symptoms and signs. Maintenance therapy and long-term surveillance are important.

2. Healthy vulvar skin expressed ERRα, ERRβ and ERRγ independent of age or hormonal status. In LS, the expression of ERRα was decreased independently of the clinical features, and showed no prognostic value. ERRα staining intensity further reduced in premalignant vulvar lesions. In vSCC, ERRα expression was lost or substantially decreased in all samples. This reduction of ERRα in cancer progression may associate with the inflammation-induced nature of vSCC.

3. Overexpression of Serpin A1 was associated with the pathogenesis of LS and vSCC. Tumor-cell specific Serpin A1 expression was detected in both LS-dependent and HPV-dependent vSCC, and the expression levels increased in a malignant progression. However, Serpin A1 immunohistochemistry or serum levels seem not to be useful as prognostic markers in LS or vSCC. p53 positivity in LS was common, and it did not predict the malignant potential in LS. p53 positivity correlated with increased Serpin A1 levels in vSCC. p16 was not detected in LS, but a coexistence of LS and p16 positivity was not uncommon.

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10 ORIGINAL COMMUNICATIONS

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Childhood Lichen Sclerosus—A Challenge for Clinicians

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Abstract: **Childhood lichen sclerosus (LS) is a rare and often misdiagnosed inflammatory dermatitis with an unpredictable course. The complications of LS are architectural changes of the vulva; malignant transformation is possible. The objective of our study was to define the background and the long-term course of childhood LS. A registery study identified 44 children with LS treated at Tampere University Hospital, Tampere, Finland, from 1982 to 2010. A questionnaire was sent to the identified patients and 15 responded. The clinical depiction of LS varied significantly. LS was diagnosed in only 16% of the patients at the referring unit. Autoimmune disorders were observed in 6 of the 44 patients. High prevalences of Turner's syndrome (2/44) and kidney disease (2/44) were noted. The majority of the patients were treated with topical corticosteroids. Eight developed architectural changes of the vulva. The questionnaire revealed that three of six patients who were asymptomatic at the end of the registery study follow-up experienced a recurrence of symptoms. None of them were undergoing follow-up. Nine of the 15 patients reported reduced quality of life. Childhood LS is a heterogeneous disease with a remarkable effect on quality of life. The misdiagnosis of childhood LS is common. The association between LS and autoimmune diseases should be noted. The high prevalence of Turner's syndrome raises questions regarding the influence of low estrogen levels on the development of LS. The prognosis cannot be predicted, so long-term follow-up is recommended. New tools for diagnosis and surveillance are needed.**

Lichen sclerosus (LS) is a type of a chronic inflammatory dermatitis; 5% to 15% of cases of LS occur in prepubertal girls (1,2). The estimated prevalence in girls is 1 in 900 (3). The clinical variability and rarity of childhood LS makes it a diagnostic challenge. Before an accurate diagnosis of

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LS is made, infections, trauma, or sexual abuse may be suspected (4). Other differential diagnoses are allergic or irritative dermatitis, vitiligo, lichen planus, and morphea (2).

The average age of LS onset in girls is 4 to 6 years, and the diagnostic delay is 1 to 2 years on average (3,5–10). LS most often manifests in the anogenital area, but 15% of patients have extragenital lesions (11). The clinical variation of LS lesions is wide. The typical presentation involves polygonal papules with central indentation, which coalesce over time and form ivory-white, atrophic, fragile plaques (1,11). Edema, purpura, erosion, fissures, or submucosal hemorrhage can also be detected (1,11). Lesions can appear at the site of trauma, a response known as the Koebner phenomenon (2). The histology of LS lesions is unambiguous and similar in all age categories (12).

The most common LS symptoms include anogenital itching, soreness, constipation, and dysuria, but it can be asymptomatic (1–3,6,10). The course of the disease is unpredictable; the complete disappearance of LS lesions is rare, and relapses are common (12,13). Anogenital structural changes may occur as a complication of LS (11). In women, LS lesions are regarded as potentially premalignant, and transformation leading to vulval squamous cell carcinoma (SCC) occurs in approximately 5% (14). The risk of cancer in children with LS has not been established.

Studies on humoral- and T-cell-mediated immunologic responses support the hypothesis that LS is an autoimmune disease (15,16). Approximately 7% to 14% of children with LS have another autoimmune disorder, such as autoimmune thyroiditis, vitiligo, alopecia areata, celiac disease, or type 1 diabetes mellitus (3,7,12,14,17). In addition, an autoimmune disease is found in 17% to 65% of first-degree relatives of children with LS (3,12,16).

Genetic traits related to LS have been observed; 17% of pediatric patients have a positive family history of LS, and sibling pairs have been reported (3,18). Associations between LS and both human leukocyte antigen subtypes and polymorphism of interleukin-1 receptor antagonist (IL-1RA), which play roles in immunology, have been reported (11). IL-1RA gene polymorphism has also been associated with other autoimmune and autoinflammatory disorders (inflammatory bowel disease, lupus erythematosus, psoriasis, and alopecia areata) (19), although germ-line mutations specific to LS have not been identified.

The presentation of LS is bimodal during phases of low estrogen (before menarche and after menopause), although the role of sex hormones in the disease pathogenesis remains unclear (11). A few studies have shown an association between gonadal dysgenesis or Turner's syndrome and LS (20,21).

The British Association of Dermatologists published revised guidelines for the management of LS in 2010 (22). Topical ultrapotent corticosteroids are the first-line treatment. Topical calcineurin inhibitors may also be effective in anogenital LS, but their long-term risks are unknown (22). Treatments relieve symptoms and are believed to prevent the development of architectural changes in the vulva, although it is unknown whether treatments reduce the risk of malignancy (23).

Our hypothesis was that the clinical diagnosis of childhood LS is challenging for most physicians. Furthermore, we assumed that LS treatment prevents long-term complications. The aims of the present study were to define referral indications, the clinical picture, comorbidities, and the long-term clinical course of childhood LS in a hospital-based cohort. The family history of LS, the effect of the disease on patients' lives, and the prevalence of sexual abuse allegations were evaluated.

MATERIALS AND METHODS

The study consisted of two separate components. The register study focused on children with LS younger than 19 years of age who were treated at the Tampere University Hospital, Tampere, Finland, from 1982 to 2010. The data were collected using a structured protocol that focused on predisposing factors, clinical phenotype, symptoms, histopathology, therapy, LS outcome, comorbidities, and family history.

A questionnaire was sent to LS registery study patients to obtain further data on their past gynecological history, current LS symptoms and treatment, comorbidities, family occurrence of LS, and possible history of sexual abuse. The histopathologic samples were reassessed to confirm the diagnosis.

The Pirkanmaa Hospital District Ethics Committee reviewed this study plan.

RESULTS

Registery Study

Description of Study Population and Referral Indications Forty-four girls and one boy with LS were identified in our registery survey. Because of the small number of affected boys, this study focused on girls only. The population of girls younger than 19 years of age in the Tampere University Hospital area is approximately 50,000, which gives a prevalence of

1:1100 in girls. The onset of symptoms occurred on average at 7.1 years of age (range 2–18 yrs). The symptoms of LS began after menarche in only a few patients. The first visit to the hospital occurred at an average age of 9.0 years (range 3.5–18.7 yrs). The delay from symptom onset to diagnosis was 1.3 years (range 0–8 yrs).

Lichen sclerosus was accurately diagnosed in 7 girls (16%) and misdiagnosed in 37 (84%) before referral. The referral indications included nonspecific inflammation of the vulva in 10 girls (23%), dermatitis in 8 (18%), pruritus in 5 (11%), unidentified or discolored skin lesions in 4 (9%), delayed puberty in 3 (7%), and genital bleeding in 2 (5%). One girl was referred because of suspicion of sexual abuse, and suspicion of sexual abuse arose during hospital visits for two other girls. At the end of the registery study, the follow-up period ranged from 0 to 11.7 years (average 2.6 yrs).

LS Symptoms and Signs Seven of the 44 patients were asymptomatic. The most common LS symptoms and signs are presented in Tables 1 and 2. Figure 1A depicts a hemorrhagic LS lesion in one of our patients. Lesions were located in the genital area in 40 girls (91%). Eight girls (18%) had extragenital LS lesions, four of whom had genital and extragenital lesions. The extragenital lesions of the girls with genital and extragenital lesions were numerous and located on the trunk and extremities. Their disease also remained active at the end of the registery study follow-up period. In the four girls with only extragenital lesions, the number of lesions varied.

Anogenital infection was diagnosed in 15 patients (34%): *Streptococcus agalactiae* in 4, beta-hemolytic streptococci in 4, *Streptococcus pyogenes* in 2, coagulase-negative staphylococci in 3, and *Staphylococcus aureus* in 1. Human pinworms were detected in six patients (14%).

TABLE 1. *Symptoms of Lichen Sclerosus*

Symptom	Number of patients	%
Vulval itching	31	70
Dysuria	19	43
Pruritus ani	7	16
Constipation	5	11

TABLE 2. *Signs of Lichen Sclerosus*

Sign	Number of patients	%
Ivory-white skin	40	91
Genital erosion	32	73
Purpura	25	57
Hyperkeratosis	23	52
Lichenification	13	30
Atrophy	10	23
Disturbance of the vulvar architecture	8	18
Vesicle, bullae	5	11

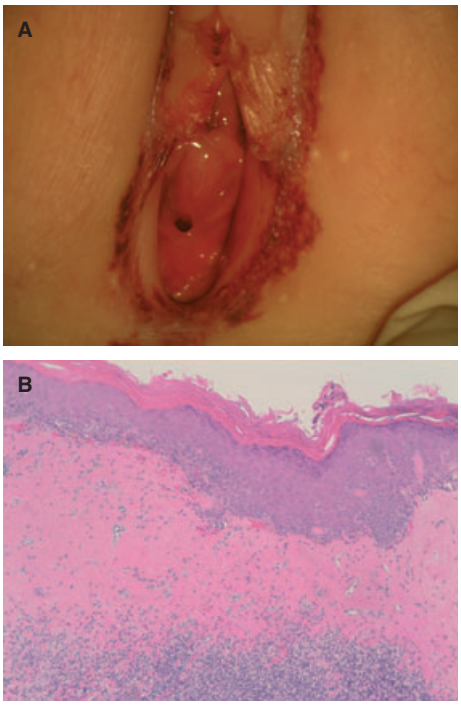

Figure 1. (**A**) A 7-year-old girl with hemorrhagic LS lesions at Hart's line and the labia minora. Lichenification of the perineal skin and lichenification with hyperkeratosis at the posterior fourchette were observed. (**B**) Typical histology of LS: hyperkeratosis, some basal vacuolar changes, homogenization of the collagen, and a lymphocytic infiltrate (hematoxylin and eosin; original magnification 100×).

LS Histology LS was histologically verified in 29 patients (66%). Biopsy samples typically showed hyperkeratosis (59%), lymphocyte infiltration (57%), and hyalinization (52%) (Fig. 1B). Reevaluation of the samples (26 of 29 available) confirmed the diagnosis.

LS Treatment Forty-two patients (95%) were treated with topical corticosteroids, and potent or ultrapotent topical corticosteroids were used in 38 (86%). Calcineurin inhibitors (tacrolimus and pimecrolimus) were used in seven patients (16%), of whom four had architectural vulval changes. Vitamin A ointment, dexpanthenol, and antimycotic and antimicrobial ointments were occasionally used. One girl with genital LS was treated with a systemic retinoid (etretinate).

LS Comorbidity Six girls (14%) had an autoimmune comorbidity: morphea in two, vitiligo in two, granuloma

annulare in one, and celiac disease in one. Ten girls (23%) had an atopic disease (atopic eczema, allergy, or allergic asthma). Two girls (5%) had Turner's syndrome, one of whom had a horseshoe kidney. Two other girls had kidney disease (polycystic kidney disease and nephrotic syndrome). One had delayed puberty of unknown etiology.

Questionnaire Study

Fifteen girls with LS (34%) completed the questionnaire (Table 3). At the time of the survey, their ages ranged from 7 to 29 years. The LS symptoms had appeared 3 to 23 years earlier, and the time from the first hospital visit to the survey ranged from 2 to 21 years. Nine of the 15 patients had active LS at the end of the registery study follow-up. Four of these nine had active LS, and five were asymptomatic at the time of the questionnaire. Three of the six patients who were asymptomatic at the end of the registery study reported recurrence of symptoms.

Nine patients (60%) were not undergoing follow-up at the time of the survey. Patients with active LS used topical corticosteroid treatment. None of the 15 patients reported sexual abuse. To the question "Has LS reduced your quality of life?" 10 (67%) answered yes. None reported human papilloma virus or herpes simplex infections.

LS and Family History The registery and questionnaire studies showed a positive family history of LS in four patients (9%). One patient reported LS in a first-degree relative (sister) and three in second-degree relatives (grandmothers).

DISCUSSION

Childhood LS is a challenge for clinicians. In the present study only 16% of the individuals with LS were accurately diagnosed upon admission. The unfamiliarity and rareness of this disease is probably the cause of misdiagnosis. In our study, the prevalence of LS was 1:1100 in girls, a lower prevalence than previously reported (4), although LS is most likely an underdiagnosed disease. In our study, the diagnostic delay was 1.3 years on average, which may be inconvenient and frustrating to patients and their families. Seventy percent of study patients reported vulval itching, 43% reported dysuria, and 16% reported pruritus ani, in accordance with previous studies (1,2,8,10,22). Only 11% of our patients had constipation, which has been reported in up to 67% of patients in previous studies (3,7,8,10). The clinical

TABLE 3. *Data Obtained from the Questionnaire*

Patient	Age at onset (yrs)	Interval from initial symptoms to query (yrs)	Age in 2011 (yrs)	Menarche (yrs)	Genital lesions	Extragenital lesions	Comorbidity	LS at the end of follow-up or in 2010	Symptoms at time of query (2011)	Reduced quality of life	Follow-up continues
1	3	23	26	NA	+	−	Atopy, depression	Active	−	−	−
2	4	17	21	15	+	−	Atopy	Remission	−	−	−
3	7	15	22	13	+	−	Endometriosis, hypothyroidism	Active	+	+	+
4	9	15	25	13	+	−		Remission	+	+	−
5	15	14	29	13	NA	+		Active	−	+	−
6	6	12	18	11	+	−	Atopy	Active	−	−	−
7	2	7	9	Prepubertal	+	−	Atopy	Active	+	+	+
8	4	7	12	11	+	−		Active	+	+	+
9	9	5	14	12	NA	+	Morphea	Remission	−	+	+
10	5	5	10	Prepubertal	+	−		Remission	+	+	−
11	7	4	11	Prepubertal	+	−		Active	−	+	+
12	14	4	18	17	+	−	Delayed puberty	Remission	−	−	−
13	3	3	7	Prepubertal	+	−		Remission	+	+	−
14	5	3	8	Prepubertal	+	−	Atopy, vitiligo	Active	+	+	+
15	12	3	15	13	+	−	Morphea	Active	−	−	−

NA, not available.

presentations were ivory-white lesions in 91% of cases, genital erosion in 73%, purpura in 57%, hyperkeratosis in 52%, and distortion of the vulval architecture in 18%, as has been documented previously (3,7) (Table 2). These symptoms and signs should guide doctors to consider the possibility of LS.

More than one-third of our patients presented with a concurrent bacterial infection in the genital area. Our study is consistent with previous studies that found that symptomatic genital infections (*Streptococcus* or *Staphylococcus*) are common in children with LS (3,4,11). Especially in these patients, an LS diagnosis might not be made without follow-up examinations.

The level of distress in children with LS is remarkable; 67% of our patients reported reduced quality of life. This is the first study of the quality of life in children with LS, and other structured questionnaires will be needed to obtain more precise details.

Child sexual abuse was suspected in three girls (7%), but none disclosed any sexual abuse. For clinicians unfamiliar with childhood LS, genital lesions that mimic trauma may lead to suspicion of sexual abuse (11). In previous studies, sexual abuse was suspected in 14% to 70% of children with LS (2,7,10). Childhood LS is diagnosed in only 4% of sexual abuse cases (24). The higher frequencies of suspicion in previous studies may indicate cultural or educational differences or the initial difficulty of diagnosing childhood LS, but it should be kept in mind that LS and sexual abuse may coexist because LS can occur at the site of trauma (4).

LS is considered to be an autoimmune disease (21). In concordance with previous studies, 14% of our patients had autoimmune disease comorbidity (3,8,15). This percentage is higher than the prevalence of autoimmune diseases in the general Finnish population, which is approximately 5% to 9% in adults (25). In children, the prevalence is considerably lower (e.g., the prevalence of autoimmune thyroiditis and celiac disease is estimated to be 1%, and the prevalence of rheumatoid arthritis is thought to be 0.1%) (26). Thus autoimmune diseases should be considered in the follow-up of LS patients.

Two girls in our study had Turner's syndrome. Previously only a few publications have reported an association between gonadal dysgenesis or Turner's syndrome and LS (20,21). In patients with Turner's syndrome, the frequency of coincident autoimmune disease is high, which may explain this association (27). In addition, Turner's syndrome is associated with low estrogen levels. This association raises questions regarding the influence of low estrogen on the development of LS, given that the dual peak ages of LS incidence is already known (11). In most patients included in our study, the onset of disease was before menarche (mean age 7.1 yrs), but no studies have shown a link between LS and low estrogen levels, and further studies are needed (11).

Two of our patients had kidney disease, and one other patient had a structural kidney malformation (a horseshoe kidney), which can be explained by her coincident Turner's syndrome. The association between LS and kidney disease has not been reported in recent studies. A study from 1967 reported a high prevalence of kidney disease in children with LS; in a group of 24 children, there was 1 with nephrosis, 2 with a congenital defect of the genitourinary tract, and 3 with recurrent genitourinary tract infections (28). This possible association requires further study.

One previous study reported a positive family history in 17% of children with LS (3). Four of our patients (9%) had a family history of LS. Two patients had genital and extragenital LS lesions and the co-occurrence of another immunologic disease: atopy in one and vitiligo in other. The association between extragenital and genital LS, immunologic disease, and familial LS has not been reported previously. Our speculation is that these patients have some heritable genetic variation that predisposes them to immunologic diseases. Previous studies have shown associations between LS and human leukocyte antigen subtypes and between LS and IL-1RA polymorphism (11). Our findings warrant the recruitment of families with LS for further analysis to identify a possible germ-line variation or mutation predisposing to LS. The characterization of heritable variations or mutations in LS with the newest molecular biology techniques such as whole-genome single-nucleotide polymorphism, copy number variation analysis, and exome sequencing could offer new insights into the etiology of LS.

At the end of our registery study follow-up, 43% of patients were asymptomatic and 57% had symptoms and signs of LS, results that are consistent with those of earlier follow-up studies of childhood LS (Table 4). The course of childhood LS is unpredictable. An important finding is that, according to the questionnaire, three of the six asymptomatic patients experienced a recurrence of symptoms, and none were undergoing follow-up. Although none of our patients developed vulval SCC during the retrospective follow-up period of 2 to 21 years, there is one case report of an individual with childhood-onset LS who developed vulval SCC at 32 years of age (1).

Despite the use of topical steroid treatment in 95% of the patients, architectural changes in the vulval

TABLE 4. *Previous Follow-Up Studies*

Study	*n*	Country	Patient age, years	Length of follow-up	Symptomatic at the end of follow-up, *n*
Lichen sclerosus et atrophicus in children (Loening-Baucke (5))	10	United States	3–11	3 months to 3.3 years	7
Lichen sclerosus et atrophicus—a review of 15 cases in young girls (Berthe-Jones et al (8))	15	United Kingdom	2–15	Some years, no exact data provided	14
Lichen sclerosus et atrophicus in children and young adults (Helm et al (12))	52	United States	3–30	Maximum 13 years, average 10 years	29
Genital lichen sclerosus in childhood and in adolescence (Ridley (9))	37	United Kingdom	2–13	Months to 14 years	36
Childhood vulvar lichen sclerosus: an increasingly common problem (Powell and Wojnarowska (3))	70	United Kingdom	2–16	Maximum 9 years	16 of 18 who reached puberty
Childhood lichen sclerosus. The course after puberty (Powell and Wojnarowska (13))	21	United Kingdom	Adults	Linked to study immediately above	16
Pimecrolimus 1% cream for anogenital lichen sclerosus in childhood (Boms et al (6))	4	United Kingdom	4–9	3–4 months	NA
Childhood onset vulvar lichen sclerosus does not resolve at puberty: a prospective case series (Smith and Fischer (1))	12	Australia	3–18	Maximum 10 years	9
Childhood lichen sclerosus is a rare but important diagnosis (Jensen and Bygum (10))	36	Denmark	1–18	Maximum 2.9 years	NA

NA, not available.

area were observed in 18% of our patients. Half of the patients with architectural changes were also treated with calcineurin-inhibitor ointments. Present international guidelines recommend that topical calcineurin inhibitors should not be used as a first-line treatment because of the potential carcinogenic effect of these drugs (22). Our study shows that even effective treatment of LS may not prevent architectural changes. Because of the complications of LS, long-term follow-up is highly recommended.

We conclude that childhood LS has a great effect upon the lives of patients and their families, decreasing quality of life. The clinical course of pediatric LS is unpredictable, so long-term follow-up is recommended. Further studies are needed to clarify the genetics of LS and the possible links between low estrogen levels, kidney disease, and LS.

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Reduction in ERRα is associated with lichen sclerosus and vulvar squamous cell carcinoma

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HIGHLIGHTS

- The cytoplasmic expression of ERRα is substantially decreased or lost in vulvar SCC.
- LS and VIN lesions show diminished ERRα staining in relation to normal vulvar skin.
- The role of ERRα as a prognostic marker remains questionable.

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ABSTRACT

Objective. ERRs (estrogen-related receptors) regulate energy metabolism, the cell cycle and inflammatory processes in both normal and cancer cells. Chronic inflammation induced by lichen sclerosus (LS) or human papilloma virus (HPV) precedes vulvar squamous cell carcinoma (vulvar SCC). We investigated the expression of ERRα, ERRβ and ERRγ in normal vulvar skin, LS as well as LS-dependent and LS-independent/HPV-related vulvar SCC.

Methods. A total of 203 samples were analyzed for ERRα, ERRβ and ERRγ by using immunohistochemistry. These included 37 normal vulvar skin samples, 110 LS samples, 6 vulvar intraepithelial neoplasia (VIN) samples and 50 vulvar SCC samples.

Results. A substantial reduction in or disappearance of ERRα was detected in all vulvar SCC samples. A total of 79% of childhood-onset LS and 51% of adulthood-onset LS lesions showed decreases in ERRα staining. A gradual reduction in ERRα cytoplasmic staining was observed from healthy vulvar skin to precursor lesions and further to SCC. Nuclear ERRα staining was observed in 8/33 (24%) LS-dependent and 10/17 (59%) LS-independent SCC samples.

Conclusions. ERRα, a key regulator of cell energy metabolism, may play a role in the pathogenesis of both LS and vulvar SCC.

1. Introduction

Lichen sclerosus (LS) is a chronic autoimmune dermatitis that often affects the genital area. In females, LS has a dual-peak incidence at the times of low estrogen production [1]. Post-menopausal prevalence is approximately 1:300, and pre-puberty prevalence is approximately 1:900 [2,3]. However, the role of estrogen in LS has remained ambiguous [4]. LS is a potentially pre-malignant state; 4–7% of women with LS will develop vulvar squamous cell carcinoma (SCC) [5].

Carcinoma of the vulva is relatively rare and involves 4% of gynecological malignancies. SCC represents over 90% of all vulvar cancer types [6]. LS and chronic human papilloma virus (HPV) infection are two frequent precursors of vulvar SCC. However, their courses to vulvar SCC differ according to precursors (*differentiated* VIN (*d*VIN) for LS-dependent and *usual* VIN (*u*VIN) or *classic* VIN for HPV-dependent), histological characteristics and protein markers [7]. However, no difference in survival has been found [8].

Estrogen-related receptors (ERRs), including ERRα, ERRβ and ERRγ, are a family of nuclear transcription factors (American nomenclature committee NR3B1, NR3B2, and NR3B3, respectively) [9]. ERRs were discovered due to their structural similarity to estrogen receptors (ERs), which mediate the effects of estrogen in tissues. ERRs participate in the estrogen signaling pathway due to similarities with ERs in regard to structure, binding sites, co-activators and target genes; however, ERRs do not bind estrogen itself [10]. Notably, a majority of ERR target genes do not participate in classical ER signaling [11]. ERRs regulate the transcription of numerous genes that affect metabolism and the cell cycle and play major roles in mitochondrial respiration [12,13].

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ERRα regulates genes involved in lipid uptake, mitochondrial biogenesis, fatty acid oxidation and glucose metabolism, especially in tissues with high energy demands [9]. ERRγ target genes are similar to those for ERRα; however, regulation of these genes can have contradictory effects [13]. ERRβ is necessary for, e.g., placental formation and nervous system development. Recent studies also indicated that ERRβ plays a role in stress response [9,14]. ERRs have been studied in various cancer types and discovered to be important prognostic factors for breast, ovarian and colon cancer, among others [15]. ERRs also regulate immunology involving T-cell differentiation and macrophage activation [16,17].

ERRs are linked to estrogen signaling, tumor progression, metabolism and inflammatory processes. To our knowledge, the role of ERRs in vulvar skin, LS and SCC has not been previously studied. Using immunohistochemistry, we assessed the association between ERRs and LS as well as vulvar SCC.

2. Material and methods

2.1. Patients

The study included LS and vulvar SCC patients visiting the Department of Gynecology or the Department of Dermatology at Tampere University Hospital from 1981 to 2014. A total of 203 biopsy and vulvectomia samples from 128 patients were included in the analysis (Table 1).

A total of 24 childhood-onset LS patients (5–19 years of age at the time of the biopsy, 20 of which were pre-menarcheal) and 51 adult vulvar LS patients (19–86 years of age at the time of the biopsy, 49 of which were post-menopausal) were included in the analysis. Seventeen of the adult vulvar LS patients also agreed to a biopsy from normal vulvar skin.

Three extra-genital LS samples were stained for comparison. These patients had no genital LS manifestations.

A total of 50 vulvar SCC patients who were 39–90 years of age were included in the study. Thirty-three of these patients had LS-dependent and 17 LS-independent vulvar SCC. From each patient we obtained a carcinoma sample. From LS-dependent vulvar SCC patients we also obtained a sample of LS-lesion. Six of the carcinoma samples showed a VIN area next to carcinoma (five dVIN, one uVIN). Twenty vulvar SCC patients also agreed to a biopsy from normal vulvar skin. The mean follow-up for vulvar SCC patients was 3.9 years.

The Ethics Committee of Pirkanmaa Hospital District approved and the Finnish National Supervisory Authority for Welfare and Health gave its official permission for the use of diagnostic histological LS and vulvar SCC samples from adults and minors. The patient register files were studied on January 2015 for disease history and were accessed with the permission of the medical head of the university hospital.

2.2. Immunohistochemistry

The stainings were performed with monoclonal anti-human ERRα, ERRβ and ERRμ antibodies (Perseus Proteomics, catalog numbers PP-H5844-00, PP-H6705-00, and PP-H6812-00, Tokyo, Japan) using a Ventana BenchMark immunostainer and a Ventanan Ultraview DAB Detection Kit (Ventana, Tucson, Arizona) according to the manufacturer's instructions. The dilutions were 1:100 for ERRα, 1:100 for ERRβ and 1:25 for ERRγ.

To assess the accuracy of the ERR stainings, the abdominal skin of a female of 40 years of age who was undergoing an obesity operation was used as a positive control.

The stainings were evaluated by a dermatopathologist (author 2) and author 1 and were graded using a scale of 0/+/++/+++ (+++ = stained as control/normal vulvar skin, ++ = moderately decreased staining, + = substantially decreased staining, basal epidermal layer stained slightly but upper epidermis unstained, 0 = unstained).

2.3. Statistical analysis

Fisher's Exact Test was used to determine the association between ERRα staining and architectural changes in the vulva. The Kruskal–Wallis Test was used to assess the association between the duration of the LS symptoms and ERRα staining. In the vulvar SCC patients, the associations with nuclear staining and survival as well as with histological grade were analyzed using a Kaplan–Meier analysis.

3. Results

In healthy vulvar skin, ERRα was detected in the cytoplasm of the epidermal keratinocytes (Fig. 1). ERRβ showed nuclear staining in epidermal keratinocytes. In the dermis, macrophages/Langerhans cells were stained. ERRγ showed both cytoplasmic and nuclear staining in the epidermis with a gradient, the basal layer was the most intensively stained. In the dermis, the sweat glands, follicles and lymphocytes/macrophages were stained. The staining of normal vulvar skin was equal among all age groups and was independent of hormonal status. The control staining of the abdominal skin showed similar results.

The results are shown in Table 1.

In childhood-onset vulvar LS, a decrease in cytoplasmic ERRα staining (graded + or ++) compared with normal vulvar skin (+++) was observed in 19/24 (79%) samples. During biopsy, 20/24 girls were premenarcheal. No association with ERRα staining and pubertal status was observed. The ERRβ and ERRγ staining did not differ between LS-lesions and normal vulvar skin.

The cytoplasmic ERRα staining of adult LS samples without progression to vulvar SCC showed a decrease (+ or ++) in 26/51 (51%) of the

Table 1
Results for different patient groups.

	Patients	Samples	ERRα, cytoplasmic staining decreased	ERRα, nuclear staining detected
LS, minors	24	24	19/24 (79%)	0
LS, adults	51	51 LS	26/51 (51%)	0
		17 NVS	0	
Vulvar SCC, LS-dependent	33	33 SCC	33/33(100%)	8/33 (24%)
		5VIN	4/5 (80%)	
		32 LS	19/32 (59%)	
		11 NVS	0	
Vulvar SCC, LS-independent	17	17 SCC	17/17 (100%)	10/17 (59%)
		1 VIN	1 (100%)	
		9 NVS	0	
Extra-genital LS	3	3	1/3 (33%)	
	128	203	120	18

SCC = squamous cell carcinoma.
LS = lichen sclerosus.
NVS = normal vulvar skin.

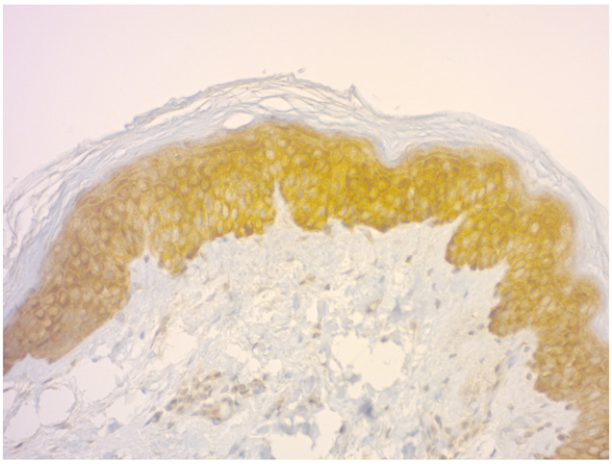

Fig. 1.. ERRα immunohistochemical staining in normal vulvar skin, cytoplasmic staining in epidermal keratinocytes (graded +++).

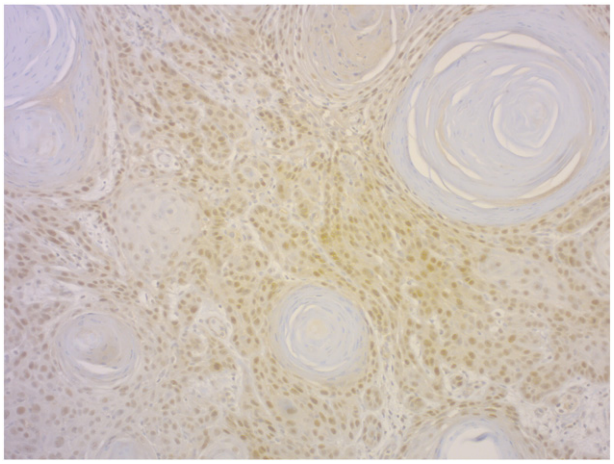

Fig. 3. ERRα immunohistochemical staining in vulvar SCC, nuclear staining (cytoplasm graded +, nuclear staining positive).

subjects compared with healthy vulvar skin (+++) (Fig. 2). Again, the ERRβ and ERRγ stainings were similar in LS-lesions and in normal vulvar skin. The ERRα stainings were compared with the clinical data, and there were no correlations between the ERRα staining and the duration of the symptoms or the architectural changes in the vulva ($p = 0.367$ and $p = 0.485$, respectively).

Regarding the extra-genital LS samples, the ERRα staining decreased in one sample (graded +) and stained in a similar manner to the control in two samples (graded +++).

A substantial reduction in or loss of cytoplasmic ERRα staining was detected in all 50 SCC samples (graded 0 or +). Nuclear staining with simultaneous ERRα cytoplasmic staining loss was detected in 8/33 (24%) LS-dependent and 10/17 (59%) LS-independent SCC samples (Fig. 3). The LS lesions in the LS-dependent vulvar SCC patients showed a relative decrease in ERRα staining compared with normal vulvar skin in 20/33 (61%) samples (graded + or ++). VIN area showed a relative decrease in cytoplasmic ERRα staining compared with normal vulvar skin in 5/6 cases and 4/6 LS lesions. The ERRα staining intensity further decreased from VIN to SCC lesions (Fig. 4).

The nuclear vs. cytoplasmic ERRα staining was compared with vulvar SCC histological grade, patient survival and FIGO staging (all patients staged according to FIGO 2009 staging). No associations were detected between nuclear staining and patient survival or FIGO staging in either vulvar SCC patient group. Nuclear staining may associate with higher histological grade; however, this finding was not statistically significant (nuclear staining for histological grade I: 27.8%, II: 37% and III: 40.0%, $p = 0.665$).

The expression of ERRβ and ERRγ in the LS and SCC lesions did not differ from that of normal vulvar skin.

4. Discussion

To our knowledge, this is the first study of ERRs in vulvar skin, LS or SCC. The ERRα cytoplasmic staining was substantially decreased or lost in all of the vulvar SCC samples. We found moderate or substantial decreases in ERRα staining in 64/110 (58%) LS lesions compared with normal vulvar skin. Similar outcomes were observed in both childhood- and adult-onset LS and regardless of the course of the disease. With LS-dependent SCC patients, the gradual reduction in ERRα staining was observed when LS progressed to VIN and further to SCC.

We demonstrated that normal vulvar skin expresses all three ERR isoforms. This staining pattern was in accordance with studies on ERR expressions in normal extragenital skin [18–20]. ERRα and ERRγ expressions were previously shown to decrease in the vagina after menopause [21]. In our study, the expressions of ERRα, ERRβ and ERRγ in the vulva were independent of age and hormonal status. Vaginal tissue differs from vulvar tissue in steroid receptor ratios: estrogen receptors dominate in the vagina, whereas there are more androgen receptors than ERs in the vulva. This may explain the differences in staining. Interestingly, LS is never detected in the vagina [1].

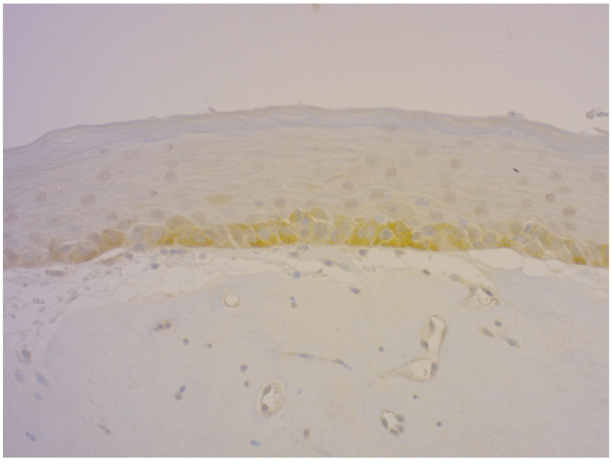

Fig. 2. ERRα immunohistochemical staining in LS, decrease in staining compared with normal vulvar skin, only cytoplasm of basal keratinocytes stained (graded +).

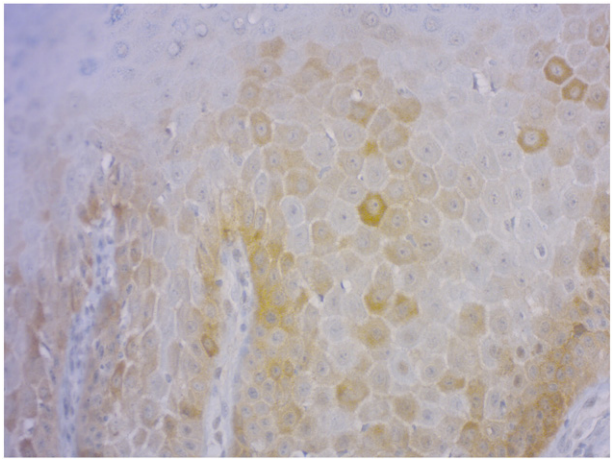

Fig. 4. ERRα immunohistochemical staining in differentiated VIN and SCC, cytoplasmic staining gradually decreases from the VIN (graded ++) area to SCC (graded+). *Original magnifications* ×200.

LS is an autoimmune disease [1]. To our knowledge, ERRα has been studied in only one other autoimmune disease, inflammatory arthritis. In accordance with our study, ERRα is also down-regulated in inflammatory arthritis [22]. ERRs are involved in the regulation of T-cell differentiation, autoimmunity and host defense; however, the studies of ERRs role in immunology are limited [16,17]. ERRα staining was decreased in 19/24 (79%) of childhood- and 26/51 (51%) of adult-onset LS samples without progression to vulvar SCC and in 59% of the LS samples in patients with vulvar SCC. We found no correlation between ERRα staining and the duration of LS symptoms or architectural changes in the vulva. Thus, a decrease in ERRα staining appears to play a role in the LS inflammatory process but has no prognostic value in evaluating the malignant potential of LS. Previously it is known that the duration and difficulty of LS are not useful indicators of its malignant potential [5]. ERRα loss can be an early event in the pathway leading to malignancy; however, the exact pathophysiological mechanism requires further study.

The substantial decrease or disappearance of cytoplasmic ERRα staining in all vulvar SCC samples suggests that ERRα is involved in the malignant process of vulvar SCC in both the LS-dependent and LS-independent pathways. In accordance with our results, ERRα knock-down promoted hepatocellular carcinoma progression [23]. As vulvar SCC, hepatocellular carcinoma is induced by chronic inflammation. ERRα was previously found to be over-expressed in breast, ovarian, endometrial, colon, prostate, adrenocortical and thyroid cancers as well as osteosarcoma [24–32]. In breast, ovarian, prostate and colon cancer, ERRα overexpression is considered a marker of poor prognosis [33]. In these cancer types, metabolic reprogramming of tumor cells by ERRα overexpression is assumed to induce tumor growth due to more efficient energy metabolism [25].

The Warburg effect may explain the ERRα decrease observed in vulvar SCC: cancer cells rely on glycolysis, especially in an inflammatory microenvironment [33]. ERRα is an important regulator of mitochondrial respiration; when ERRα is blocked, the metabolism shifts toward glycolysis [34]. Another possible explanation can be linked to reactive oxygen species (ROS), a byproduct of mitochondrial respiration [34]. In hepatocytes, ERRα knock-down and impaired ROS levels increased cell death due to necrosis instead of apoptosis, which has higher energy demands. Cell necrosis in turn induces tumor growth due to compensatory cell proliferation [23]. Third, ROS levels also control inflammatory cytokines and many key tumor suppressor genes, e.g., *p53*, *p21* and $p16^{INK4a}$ [35]. Mutations of these genes have been linked to vulvar SCC [36]. Previous studies showed a direct link between ERRα and the cell cycle inhibitor *p21*: ERRα knock-down repressed *p21* [37,38]. Thus, the loss of ERRα and reduction of ROS levels may modulate *p53*, *p21* and $p16^{INK4a}$ activity. Further studies are required to clarify the role of ERRα in the pathogenesis of vulvar SCC and other cancer types induced by inflammation.

The localization of steroid receptor expression may also play a role in cancer progression. Shifts from nuclear to cytoplasmic ERβ expression indicate poor prognosis in vulvar SCC [39]. In our study, cytoplasmic ERRα staining is observed in normal vulvar skin. Nuclear ERRα staining was observed in 8/33(24%) LS-dependent and 10/17 (59%) HPV-dependent vulvar SCC samples. The change in ERRα localization from cytoplasmic to nuclear indicates a loss of protein function. The differences observed in nuclear staining between these two patient groups may reflect the differing pathophysiological mechanisms of these two etiological pathways to SCC. In accordance with previous studies, survival in our two cancer patient groups did not differ [8]. Furthermore, nuclear staining was not associated with prognosis or FIGO staging in our patients. Nuclear staining may increase with cancer progression in terms of histological grade. However, the limited number of patients and relatively short follow-up are prone to reduce the statistical power of these findings.

5. Conclusions

A substantial reduction or loss of ERRα expression was detected in all vulvar SCC samples. ERRα reductions also involve LS; however, the utility of ERRα as a prognostic marker is questionable in this context. ERRα staining gradually decreases in LS to VIN and further to SCC; furthermore, nuclear staining may associate with the progression of cancer.

Conflict of interest statement

No conflicts of interest.

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