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ANU AALTO

Vulvodynia

Etiology, therapeutic options and impact on quality of life

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Etiology, therapeutic options and impact on quality of life

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion at Tampere University,
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ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Tampere University Hospital, Department of Obstetrics and Gynecology
Finland

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"Success is not final, failure is not fatal: it is the courage to continue that counts."

-Winston Churchill

To my family

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One chapter in my life has now come to an end. Awesome! These next paragraphs are probably the easiest ones to write:

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Conducting research is team play – I consider myself fortunate to have been able to work with such an excellent team:

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June 2020,

Anu Aalto

ABSTRACT

Vulvodynia, a chronic genital and pelvic pain condition, affects approximately 8% of women. Localized provoked vulvodynia (LPV) is the most common subtype of the condition especially among young women. The etiology of LPV is unknown, although inflammation may play a role. The efficacy of most vulvodynia treatments has not been proven in a randomized, controlled study setting.

The aim of the study was to collect and analyze retrospective data on all women diagnosed and treated for vulvodynia at Tampere University Hospital (TAUH) in 2003–2013. Among the 133 patients, symptom correlation with demographic variables, the efficacy of treatments after follow-up, and the patients' satisfaction with treatments and different professionals were analyzed. Also, a subgroup of vulvodynia patients treated surgically at TAUH in 2003–2016 was identified. Efficacy and quality of life (QoL) were compared between the surgical and non-surgical treatment protocols. In order to evaluate the etiology of LPV the patients' vestibulectomy samples (n=12) were analyzed by immunohistochemistry and compared to the vulvar samples from healthy controls (n=15). Different subtypes of estrogen-related receptors (ERRs), estrogen receptor (ER), progesterone receptor (PR) and inflammatory T-cells (CD3) were analyzed. Finally, the microbiomes of patients suffering from LPV (n=30) with those in healthy controls (n=20), using swabs collected prospectively from the vulvar vestibulum.

To summarize, combining different treatment modalities, and age under 30 years predicted better outcome in terms of pain control in vulvodynia patients. Short-term QoL was better and self-reported pain was lower after surgical treatment. However, this benefit was not maintained after a follow-up period of three years. Furthermore, LPV patients showed differential vulvar expression of ERRß as well as dissimilarities in the vulvar microbiome when compared with controls, suggesting possible hormonal and inflammation-related factors in the etiology of LPV.

TIIVISTELMÄ

Vulvodyniaa, kroonista ulkosynnyttimien kiputilaa sairastaa arviolta 8 % naisista. Paikallinen, kosketuksesta provosoituva vulvodynia (LPV) on sen yleisin alatyyppi, erityisesti nuorilla naisilla. Vulvodynian selvää syytä ei tunneta, mutta tulehdus saattaa olla yksi LPV:n laukaiseva tekijä. Vulvodynian hoitomuodoista on olemassa vain rajallisesti tietoa satunnaistetuissa, kontrolloiduissa tutkimusasetelmissa.

Tämän tutkimuksen ensimmäinen tavoite oli kerätä takautuvasti tietoa kaikista vuosina 2003-2013 TAYS:ssa vulvodynian vuoksi hoidetuista potilaista. Näistä 133 kerätystä potilaasta selvitettiin sekä vulvodynian oireiden yhteyttä eri taustatekijöihin että annettujen hoitojen seuranta-ajan jälkeistä tehoa. Myös potilaiden tyytyväisyyttä yliopistosairaalan moniammatillisessa hoitoryhmässä annettuihin hoitoihin selvitettiin. Tästä potilasryhmästä tunnistettiin myös ne LPV-alamuotoa sairastavat potilaat, jotka oli hoidettu vuosina 2003-2016 toisaalta konservatiivisilla hoitokeinoilla ja toisaalta vestibulektomialla (vulvodynian hoidoksi tehty leikkaus, joka tähtää emättimen eteisen kivuliaan limakalvon poistoon). Toisena tutkimuksen tavoitteena oli verrata takautuvasti (36 kk seuranta-ajan mediaani) näiden potilasryhmien kokemaa kipua, elämänlaatua ja annettuja hoitoa. Tutkimuksen kolmantena tavoitteena oli verrata kirurgisesti hoidettujen LPV:ta sairastavien naisten leikkauksessa poistettuja kudosnäytteitä (n=12) vapaaehtoisten verrokkien (n=15) emättimen eteisestä otettuihin näytteisiin. Näytteistä selvitettiin eri estrogeeninkaltaisten reseptoreiden (ERR alatyypit), estrogeenireseptorin (ER), progesteronireseptorin (PR) sekä tulehduksellisten T-solujen (CD3) ilmentymistä. Neljännessä prospektiivisessa, vuosina 2018-2019 emättimen mikrobiomia käsittelevässä työssä verrattiin 30 LPV:ta sairastavan naisen emättimen eteisen näytteitä 20 kontrollien emättimen eteisestä kerättyihin mikrobiominäytteeseen. Mikrobiominäytteet analysoitiin NGS-menetelmällä.

Tutkimuksen päätulokset on koottu seuraavassa. Eri hoitojen yhdistelmä ja alle 30-vuoden ikä ennusti parempaa vulvodynia-potilaiden hoitotulosta. Kirurgisesti hoidettujen LPV-potilaiden kipu ja elämänlaatu olivat leikkauksen jälkeen paremmat kuin ei-kirurgisesti hoidetuilla. Tätä eroa ei kuitenkaan havaittu enää kolmen vuoden seuranta-ajan jälkeen. LPV potilaiden kudosnäytteissä esiintyi enemmän ERRß-reseptoreita.Lisäksi emättimen eteisen mikrobiomi erosi merkittävästi terveiden

kontrolleiden kudos- ja mikrobiominäytteistä. Näiden löydösten perusteella voidaan päätellä, että hormonaalisilla ja tulehduksellisilla tekijöillä on merkitystä LPV:n etiologiassa.

TABLE OF CONTENTS

ABBREVIATIONS

Style in List of Abbreviations is TUD Abbreviations.

AV	Aerobic vaginitis
BT	Botulinum toxin
BV	Bacterial vaginosis
CBT	Cognitive behavioral therapy
CHC	Combined hormonal contraceptives
CNS	Central nervous system
CST	Community state type
DIV	Desquamative inflammatory vaginitis
DNA	Deoxyribonucleic acid
EMG	Electromyography
ER	Estrogen receptor
ERR	Estrogen-related receptor
gCBT	Group cognitive behavioral therapy
GUV	Generalized unprovoked vulvodynia
HPV	Human papillomavirus
HUCH	Helsinki University Central Hospital
IHC	Immunohistochemistry
IL-1 β	Interleukin-1 beta
IQR	Interquartile range
ISSVD	International Society for The Study of Vulvovaginal Disease
LLLT	Low-level laser therapy
LNG-IUD	Levonorgestrel intrauterine device
LPV	Localized provoked vulvodynia
LSA	Lichen sclerosus
MBL	Mannose binding lectin
MBT	Mindfulness based therapy
MCBT	Mindfulness based cognitive behavioral therapy

M-gCBT	Mindfulness based group cognitive behavioral therapy
MVP	Multidisciplinary vulvodynia program
NGS	Next-generation sequencing
NRS	Numerical rating scale
NSAID	Non-steroidal anti-inflammatory drug
OC	Oral contraceptive
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PR	Progesterone receptor
PTSD	Posttraumatic stress disorder
PVD	Provoked vestibulodynia
QoL	Quality of life
RCT	Randomized controlled trial
sEMG	Superficial electromyography
SNRI	Serotonin and norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TAUH	Tampere University Hospital
TCA	Tri-cyclic antidepressant
TENS	Transcutaneous nerve stimulation
TNF α	Tumor necrosis factor alpha
TRPV	Transient receptor potential vanilloid
VAS	Visual analog scale

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-IV.

I Aalto A, Vuoristo S, Tuomaala H, Niemi R, Staff S, Mäenpää JU. Vulvodynia - Younger age and combination of therapies associate with significant reduction in self-reported pain. Journal of Lower Genital Tract Disease. 21(3):209-214, July 2017

II Aalto A, Huhtala H, Mäenpää J, Staff S. Combination of treatments with or without surgery in localized provoked vulvodynia - outcomes after three years of follow-up. Bioresearch Open Access. 1(8):25-31, March 2019

III Aalto A, Huotari-Orava R, Luhtala S, Mäenpää J, Staff S. Expression of estrogen-related receptors in localized provoked vulvodynia. Bioresearch Open Access. 1(9):13-21, February 2020

IV Aalto A *, Mishra P *, Tuomisto S, Ceder T, Sundström K, Leppänen R, Ahinko K, Mäenpää J, Lehtimäki T, Karhunen PJ, Staff S. Secondary vulvodynia patients show reduced bacterial diversity in vestibular microbiome. (Submitted)

*equal contribution

1 INTRODUCTION

Eight percent of women are estimated to suffer from chronic pain in the genital and pelvic area, a condition known as vulvodynia (Reed et al., 2012[a]). The most frequent subtype, localized provoked vulvodynia (LPV) is the most common form of sexual pain in women under 30 years of age. Pain in LPV is located in the vulvar vestibulum and is provoked by intercourse, tampon insertion etc. The etiology of LPV remains unknown, although inflammatory, hormonal, musculoskeletal, genetic and psychological factors may contribute to its development (Pukall et al., 2016).

Inflammation is one possible etiological factor contributing to the development of LPV symptoms. Vulvovaginal infections, for example yeast infection, are known to precede pain symptoms in many LPV patients (Donders et al., 2012[a]). Various inflammatory cells are predominant in vestibular samples taken from women with LPV, although contradictory results are also obtained (Leclair et al., 2014; Tommola et al., 2015). Steroid hormone signaling is known to have modulatory effects on the inflammatory process and may thus play a role in the pathogenesis of LPV (Nadkarni et al., 2013). In addition, changes in the vaginal and vulvar microbiome can contribute to the development of certain infectious processes that may also have a role in the pathogenesis of LPV (Havemann et al., 2017). Musculoskeletal, genetic and psychological factors are also suggested to play a role in the etiology of LPV:

Treatments of LPV include local treatments and injections to the painful area, oral antidepressant or anticonvulsant medications, physiotherapy, sexual therapy and counseling, psychological interventions and psychotherapy, and surgery (Goldstein et al., 2016). Most of the conservative treatments' efficacy is similar to placebo in randomized controlled studies (Pereira et al., 2018) and no golden standard of care exists. Most of the patients receive treatment that is a combination of various treatment modalities, tailored to individual patient's need and motivation. Surgery for LPV is aimed at removing the painful area from the vulvar vestibulum and is called vestibulectomy. Vestibulectomy is relatively safe, with rare and typically only minor complications (De Andres et al., 2016). Although vestibulectomy as well as most vulvodynia treatments, have not yet been proven efficient in randomized

controlled study settings, surgery is a widely accepted treatment modality when all conservative treatments fail to provide pain relief (Goldstein et al., 2016).

Vulvodynia, being an economic burden to society (Xie et al., 2012), also seriously harms a patient's quality of life (QoL), relationships and sexuality (Xie et al., 2012). Multimodal treatments should be aimed not only to reduce the actual pain but also to concern the various aspects of a patient's life influenced by chronic pain.

The main focus of this thesis is LPV, the most common form of vulvodynia, especially among young women.

2 REVIEW OF THE LITERATURE

2.1 The vulva

2.1.1 Embryology of the vulva

During the third week of gestation, the gastrulation process establishes all three germ layers; endoderm, mesoderm and ectoderm. These layers form all tissues and organs (Sadler, 2012). These three germ layers meet at the vulva: endoderm (vestibulum from Hart's line to hymen), mesoderm (urethra and vagina) and ectoderm (labia) (Kruger et al., 2007). The cloaca is at the caudal end of the embryo. At the sixth week of development, the cloacal membrane is subdivided into the urogenital and anal membranes when the caudally growing urorectal septum fuses with the cloacal membrane. The lower third of the vagina also originates from this cloacal membrane. The cloacal folds are subdivided into the urethral folds anteriorly and the anal folds posteriorly. The anteriorly situated urogenital portion is eventually divided into separate urethral and vaginal orifices. Another pair of elevations, the genital swellings, are formed on each side of the urethral folds. In females, these swellings become the labia majora. The genital tubercle elongates to form the clitoris, where the urethral folds develop into the labia minora (Kruger et al., 2007).

2.1.2 Anatomy, histology and innervation of the vulva

The vulva consists of the labia majora, labia minora, mons pubis, clitoris, perineum and vestibule (Figure 1). The labia join anteriorly at the anterior commissure and posteriorly merge into the perineum, the anterior margin of which is the posterior commissure (Shaw et al., 2003). The innermost aspect of the vulva is the vestibule; it encompasses the openings of the urinary tract with the urethral meatus and the vagina with the hymenal ring. It is described as the part of the vulva that extends from the clitoris, posteriorly to the posterior commissure and laterally to Hart's line, where the nonkeratinized transitional squamous epithelium of the vestibule joins the

keratinized squamous epithelium at the base of the medial aspects of the labia minora (Apgar et al., 2008). The vulvar skin normally consists of cutaneous epithelium that covers the mons pubis and labia majora. It exhibits a keratinized, stratified, squamous structure with sweat and sebaceous glands, and hair follicles (Cohen Sacher, 2015). The labia minora are covered with keratinized skin containing sebaceous glands but not sweat glands or hair follicles. From the inner third of the labia minora towards the vestibulum, the epithelium changes into a nonkeratinized endodermal-originated mucosal tissue (Cohen Sacher, 2015).The nonkeratinized squamous epithelium in this area differs from the keratinized surface of the labia majora and skin elsewhere and has greater similarity to the mucous membranes of the oropharynx. The protective keratinized surface acts as a barrier and the lack of protection on the inner surface may explain why the vestibule may get irritated or infected more easily (Apgar et al., 2008). The duct openings of Skene's glands, Bartholin's glands and minor vestibular glands are also located in the vestibule.

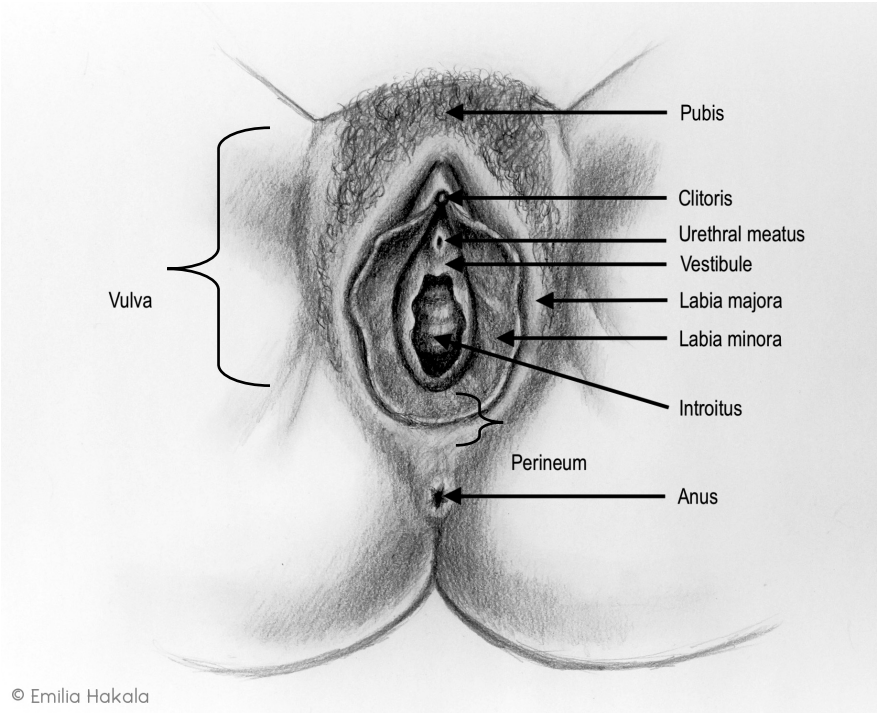

Figure 1. Vulvar anatomy

The vulva is innervated by nerve supply from the pudendal (S2, S3, S4), genitofemoral (L1-L2), ileoinguinal (L1) and ileohypogastric (T12, L1) nerves. The pudendal nerve supplies most of the superficial tissue of the vulva. The pudendal nerve can easily be damaged during pelvic surgery, operative or prolonged delivery or other traumas to the pelvic area (Danby et al., 2010). The nerve receptors in the skin specialized for pain conduction are the nociceptors, which are stimulated by heat, pressure etc. The nociceptors pass sensitization to the dorsal root ganglia (Danby et al., 2010). The nerve fibers then cross over in the spinal cord to the opposite side and up the contralateral spinothalamic tracts to the brain centers for processing. The parietal lobe of the brain recognizes the quality and location of the pain. The dorsal horn of the spinal cord controls transmission of pain signals and either permits or inhibits peripheral pain impulses (Danby et al., 2010). The descending pathway from the brain can also modulate incoming pain signals by inhibitory signals. In chronic pain overload, the neurons responsible for the downregulation of the pain can lose that ability. In chronic pain the innervated area of the skin can become hypersensitive to touch (hyperalgesic) (Danby et al., 2010).

2.2 Vulvodynia

The first report of a condition that would fulfill the criteria of vulvodynia in women is probably from the first century BC. Physician Soranus from Ephesis called it "satyriasis" back in those times. In the scientific literature it was first mentioned in 1889 by Skene (Ledger et al., 2014) and called "vulvar hyperesthesia". The next brief report was in 1928 by Kelly, who described sensitive spots in the hymenal ring that could make intercourse painful or even impossible (Ledger et al., 2014). In 1975, Weisfogel introduced the term "burning vulva syndrome" at the International Society for the Study of Vulvovaginal Diseases (ISSVD) (Petersen et al., 2008). In 1976 Pelisse & Hewitt described a syndrome of superficial dyspareunia (Ledger et al., 2014). The term vulvodynia was first proposed in the 1980's, being derived from the ancient Greek word "odynia", which means pain (Ledger et al., 2014)

2.2.1 Terminology and classification

The criteria and terminology of vulvodynia have changed several times (Petersen et al., 2008). The most recent changes to vulvodynia criteria were made at first in 2015

and more accurate definitions of pain were added in 2019 (Bornstein et al., 2016, 2019) when the 2015 Consensus Terminology and Classification of Persistent Vulvar Pain and Vulvodynia and its descriptors (2019) was published. The associated factors originally mentioned in vulvodynia classification include comorbidities and other pain syndromes, genetics, hormonal factors, inflammation, musculoskeletal factors, neurologic mechanisms (central/peripheral), psychosocial factors and structural defects (Bornstein et al., 2016). These factors related to vulvodynia acknowledge the fact that vulvodynia is probably not one disease but a constellation of symptoms of several disease processes that may overlap (Pukall et al., 2016). According to the recent criteria, the vulvodynia can be described by its location, onset, provocation and temporal pattern as described in Table 1. A clinically relevant distinction is the differentiation between localized and generalized vulvodynia (Table 1).

Table 1. The Descriptors of Vulvodynia

Onset		
	Primary	Onset of the symptoms occurs with first provoking physical contact
	Secondary	Onset of the symptoms did not occur with first provoking physical contact
Location		
	Localized	Involvement of a portion of the vulva, such as the vestibule (vestibulodynia), clitoris (clitorodynia) etc.
	Generalized	Involvement of the whole vulva
Provocation		
	Provoked	The discomfort is provoked by physical contact
	Spontaneous	The symptoms occur without any provoking physical contact
Temporal pattern		
	Persistent	The condition persists over a period of at least 3 months (Symptoms can be constant or intermittent)
	Constant	The symptoms are always present
	Intermittent	The symptoms are not always present
	Immediate	The symptoms occur during the provoking physical contact
	Delayed	The symptoms occur after the provoking physical contact

Modified from Bornstein J, Preti M, Simon J et al (2019). Descriptors of vulvodynia: A multisocietal Definition Consensus (International Society for the Study of Vulvovaginal Disease, the International Society for the Study of Women Sexual Heath, and the International Pelvic Pain Society). J Low Genit Tract Dis 23(2):161-163.

Vulvodynia is called primary if the pain is presented during the first attempted intercourse, tampon insertion etc. In secondary vulvodynia women have had a pain-free period of intercourse, tampon insertion etc. before developing symptoms (Table 1).

2.2.2 Diagnosis

Vulvodynia is defined as vulvar pain with no identifiable reason for a duration of at least three months. It may present with associated factors (Bornstein et al., 2019). A diagnosis of vulvodynia is typically made during a clinical examination. First, the vulvar area is inspected carefully. Other possible causes of pain are ruled out and a punch biopsy can also be used in differential diagnosis. There are no typical histological findings associated with vulvodynia. According to the most recent classification of vulvodynia (Bornstein et al., 2016), if, for example, some dermatosis is diagnosed, then the pain is thought to relate to that, not vulvodynia.

Differential diagnoses are listed in Table 2. Thus, vulvodynia is a diagnosis of exclusion even if it can have associated factors. The vulvar area is gently palpated with a moisturized cotton swab (Q-tip test, Figure 2) and the patient is asked to report the pain associated with touch (Sadownik, 2014). A visual analog scale (VAS) can be a helpful tool in reporting the pain. Because the vestibulum is typically a sensitive area, mild sensations (1-3/10) can be graded as normal (Sadownik, 2014). Women with localized provoked vulvodynia (LPV) typically report increased pain sensitivity to a light touch during a cotton-swab test (Sadownik, 2014). Friedrich's classical criteria for LPV are the most commonly used diagnostic criteria for the condition (Petersen et al., 2008). These criteria are:

1) The presence of pain on pressure to the vestibule or when attempting to insert an object into the vagina

2) Pain on pressure to the vestibule upon examination

3) Vestibular erythema

(Friedrich, 1987). Vestibular erythema is not mandatory to confirm the diagnosis.

Table 2. Vulvar pain caused by a specific disorder	
Infection	E.g. recurrent candidiasis, herpes
Inflammatory	E.g. dermatoses
Neoplastic	E.g. Paget's disease, squamous cell carcinoma
Neurologic	Post-herpetic neuralgia, nerve compression or injury
Trauma	E.g. female genital mutilation, obstetrical trauma
Iatrogenic	E.g. post-operative, chemotherapy, radiation
Hormonal deficiency	E.g. lactational amenorrhea

Modified from Bornstein J et al (2016). 2015 ISSVD, ISSWH and IPPS consensus terminology and classification of persistent vulvar pain and vulvodynia. J Low Genit Tract Disease 20(2): 126-130.

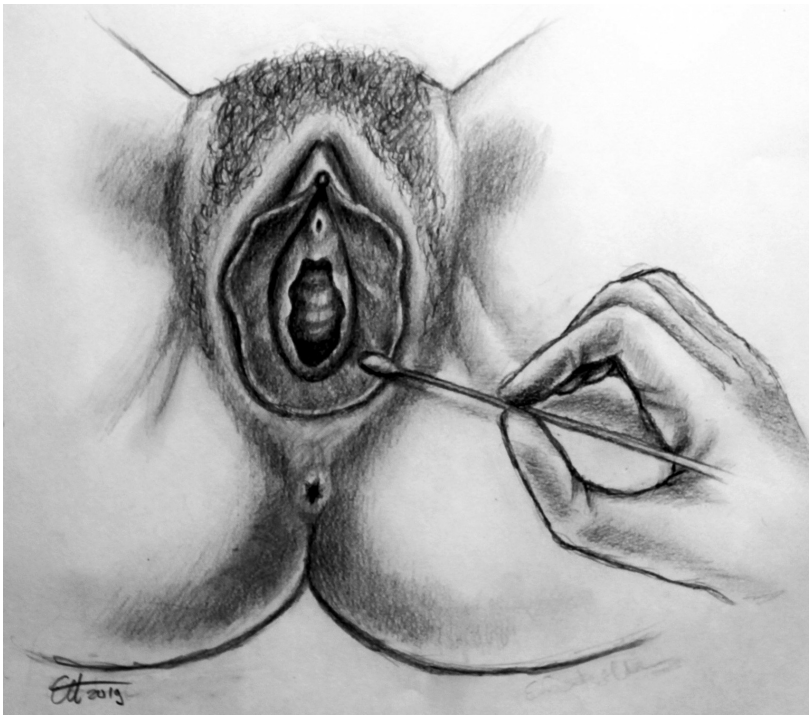

Figure 2. Q-tip test

A vulvagesiometer is a device that standardizes the amount of pressure applied to the vestibule to quantify levels of sensitivity, which is important when assessing, for example, research outcomes (Pukall et al., 2004). Cotton-swab intensity ratings may vary. At the moment, vulvagesiometers are not in clinical standard use but are mainly used in various research settings.

Coital pain (dyspareunia) is experienced in all cases of LPV. However, this type of pain is somewhat difficult to assess in a clinical setting. The tampon test has been devised to mimic coital pain (Foster et al., 2009). That test has been widely studied; it has been shown to have a good validity and can be considered to be an appropriate outcome measure in vulvodynia research (Foster et al., 2009).

2.2.3 Localized Provoked Vulvodynia and Generalized Vulvodynia

The most common form of vulvodynia is LPV (Ledger et al., 2014). In the literature, the condition is also called provoked vestibulodynia (PVD). Its former names, vulvar vestibulitis syndrome, focal vulvitis and vulvar adeninitis refer to the same condition as well (Pukall et al., 2016). LPV is also the most common form of sexual pain in women less than 30 years of age (Moyal-Barracco et al., 2010).

Generalized Unprovoked Vulvodynia (GUV), formerly called ”essential vulvodynia”, is the other main subtype of vulvodynia. In contrast to LPV, the pain in GUV is not provoked by Q-tip tests but is present all the time. The etiology of GUV is unknown but it might result from centralized pain or pudendal neuralgia (Danby et al., 2010). “Central pain sensitization” is caused by prolonged activation of the nerve fibers in the dorsal root ganglion and chronic release of neuroactive substances (Danby et al., 2010). Central sensitization refers to an increase in the excitability of neurons within the central nervous system (CNS). For example when a wound has healed, even a light touch of the area results in pain and the sensation of pain can be felt also in the surrounding skin of the wound (Danby et al., 2010).

2.2.4 Prevalence of vulvodynia

In population based studies from the United States and Sweden, the prevalence of vulvodynia is estimated to be 8.3-9.3% in the general population (Danielsson et al., 2003; Reed et al., 2012$_a$), and up to 15 % in a private gynecological outpatient clinic (Goetsch, 1991). More than 25% of women will be affected by vulvodynia at some point of their lifespan (Reed, 2012). The prevalence of vulvodynia seems to remain stable until 70 years of age and decline thereafter (Reed et al., 2012$_a$).

In one report, a majority of patients (57%) with LPV experienced their first symptoms under the age of 30 years (Arnold et al., 2006) and 20 % had their first vulvodynia symptoms under the age of 20 (Arnold et al., 2006). In a population-based study of 441 women with vulvodynia symptoms, 51% reported remission of

symptoms in 6-30 months after the initial positive assessment and only 9.7% had persistent symptoms (Reed et al., 2016).

2.2.5 Etiology of localized provoked vulvodynia

The majority of published studies focuses on etiology of LPV, GUV being much less studied. Therefore, from now on, the review of this thesis mainly focuses on etiology of LPV. Studies that have included also GUV patients are mentioned separately. If in the reviewed original study the general term 'vulvodynia' has been used with uncertainty of which subtype is concerned, then the term 'vulvodynia' is used. Several factors are suggested to involve in the development of LPV (Figure 3).

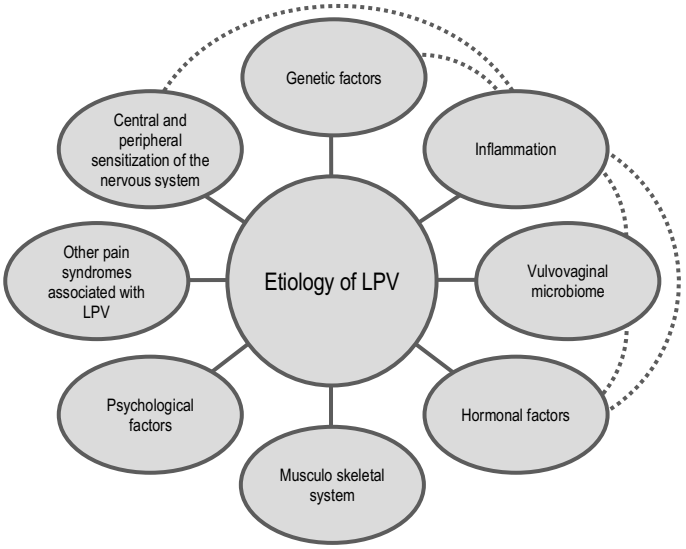

Figure 3. Etiology of LPV.

2.2.5.1 Inflammation

Inflammation is one underlying pathogenic mechanism that might lead to the development of LPV. In a study by Donders et al., more than 70% of LPV patients reported a yeast infection preceding LPV symptoms (Donders et al., 2012). Also, histories of bacterial vaginosis (BV) (Edgardh et al., 2007), trichomoniasis, genital warts and urinary-tract infections have been associated with the development of vulvodynia (Nguyen et al., 2009). A study of 231 LPV women showed that those with VAS pain scores > 7 had more aerobic vaginitis detected by microscopy than those with lower pain score. Candida species, on the other hand, were found less commonly in the most severe cases (Donders et al., 2018).

The results of studies on various inflammatory cells in cases of LPV, involving immunohistochemistry (IHC) and inflammatory mediators, are inconsistent. In one study, numbers of B-cells were similar in LPV and control patients (Leclair et al., 2014), while another study (Tommola et al., 2015) revealed higher levels of B-cells. Further, Tommola et al. (2015) found mature mucosal IgA plasma cells with a difference in B- and T-cell arrangement in germinal centers in the vulvar vestibulum in cases of LPV. Numbers of T-cells in vestibular tissue were similar in cases and controls in a study by Tommola et al. (2015), while Leclair et al. (2014) found greater numbers of CD4-positive T-cells and fewer CD8-positive cells in LPV patients compared with healthy controls (Leclair et al., 2014; Tommola et al., 2015). Levels of inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interleukin-1-beta (IL-1β) have found to be elevated in women with LPV (Foster and Hasday, 1997) compared with controls. In LPV patients, fibroblasts have been found to be extremely sensitive to C. Albicans, even in low infectious doses (Falsetta et al., 2015). This fibroblast mediated inflammatory response has shown to contribute to the development of vulvar pain (Falsetta, 2015). However, according to a systematic review of 18 studies including 400 women, only limited and contradictory evidence has been presented for the association of systemic or local inflammation with LPV (Chalmers et al., 2016).

2.2.5.2 Vulvovaginal microbiome

The microbiome has shown to be essential for physiology, nutrition and immunity (reviewed by Smith & Ravel, 2017). The vaginal microbiome is under constant change from puberty until menopause, with changing hormonal status (Smith & Ravel, 2017). In addition, the antimicrobial medication, sexual activity and menses

affect the vaginal flora (Paavonen and Brunham, 2018). Based on vaginal microbiome studies in reproductive-aged women, at least five major types of vaginal microbiota called community state types (CSTs) exist (Smith et al., 2017) (Table 3). For example, CST-IV is known to dominate the vaginal microbiome in desquamative inflammatory vaginitis, DIV (also known as aerobic vaginitis, AV) (Donders et al., 2018; Paavonen and Brunham, 2018).

Table 3. Five major types of vaginal microbiota (Community state types, CSTs)

Type	Dominated by
CST I	Lactobacillus crispatus
CST II	Lactobacillus gasseri
CST III	Lactobacillus iners
CST IV	Including species of the genera Gardnerella, Atopobium, Mobiluncus, Prevotella and other taxa in the order Clostridiales
CST V	Lactobacillus jensenii

Modified from Review of Smith and Ravel (2017) The vaginal microbiota, host defence and reproductive physiology. The Journal of Physiology 595(2), 451–463.

Vaginal cultures from healthy controls, analyzed by means of real-time polymerase chain reaction (PCR), showed the presence of *Lactobacillus crispatus*, which was not present in samples from women with symptomatic LPV or LPV in remission, demonstrating the presence of *Lactobacillus gasseri* (Ventolini et al., 2013). The researchers hypothesized that the alteration in the vaginal flora might mark the initiation of the inflammatory process that results in abnormal cytokine production and development of vulvodynia symptoms (Ventolini et al., 2013).

The microbiome of the vulvar vestibulum is much less studied compared to the vaginal microbiome. Jayaram et al. found that the dominant bacteria of the vagina closely paralleled the dominant genera of the vulva, leading to the conclusion that vaginal secretions are an important source of bacteria in the vulvar vestibulum (Jayaram et al., 2014). Women with LPV had slightly more *Streptococcus* and *Lactobacillus iners* in their samples, but major differences were not found, as analyzed by using the 16S rRNA technique (Jayaram et al., 2014).

2.2.5.3 Hormonal factors

The role of steroid-hormone activity in vulvodynia, namely estrogen and progestin signaling, is controversial. The previous studies are presented in Table 4.

Estrogen has been shown to modulate the immune response by restricting neutrophil accumulation to the site of inflammation, attenuating the release of pro-inflammatory mediators, and regulating estrogen receptor (ER) gene expression in T-, B- and dendritic immune cells (reviewed by Nadkarni & McArthur, 2013).

Table 4. Studies on hormone receptors in LPV

Study	Aim of the study	Study population	Methods	Results
Johannesson et al. (2008)	Steroid receptor expression (ER alpha and beta, progesterone receptors A and B, glucocorticoid receptor, androgen receptor and proliferation marker Ki67) and morphology in LPV	Prospectively collected biopsies 14 LPV pts+25 healthy controls	IHC	ER alpha more pronounced in LPV pts, no difference in ER beta and PR receptors or any other receptor or Ki67
Goetsch et al. (2010)	Hormone receptors (ER alpha and beta, androgen, progesterone receptors), nerves, inflammation and mast cells in LPV patients	10 primary LPV+10 secondary LPV+ 4 healthy controls. Prospectively collected samples.	IHC	No difference in hormone receptors. Nerve densities higher in primary LPV, secondary LPV had more lymphocytes than primary LPV in tender sites. Mast cells increased in tender sites compared with non-tender and healthy controls.
Leclair et al. (2011)	Differences by IHC in primary and secondary LPV	Archived vestibulectomy specimens 42+44	IHC	ER alpha, progesterone receptor nuclear immunostaining, neural hypertrophy and hyperplasia more pronounced in primary LPV
Leclair et al. (2013)	Differences in postmenopausal and premenopausal LPV (inflammation, nerves, mast cells, Estrogen receptor alpha and PR)	Retrospectively analyzed 21 postmenopausal LPV pts +88 premenopausal LPV pts (42 primary + 46 secondary)	IHC	No difference in ER alpha, less PR and neural hyperplasia in postmenopausal LPV patients than primary LPV. Also, less PR and similar neural hyperplasia compared with premenopausal secondary cases.

The role of oral contraceptives (OCs) in vulvodynia is unclear. Early onset (< 16–18 years) (Bouchard et al., 2002; Harlow et al., 2008) and a longer period of OC use have been shown to increase the risk of vulvodynia (Bouchard et al., 2002; Sjöberg et al., 1997). The risk was higher with high progestogenic, high androgenic and low estrogenic potency pills (Bouchard et al., 2002). However, the risk of new-onset vulvodynia was not shown to increase among women under 50 years of age (n = 645) using OCs (Reed et al., 2013). Women with vulvodynia had less likely used OCs prior to the onset of pain (60.7%) than those without vulvodynia (69.3%) (Reed et al., 2013).

2.2.5.4 Genetic factors

Genetic polymorphisms may affect the immune system's response to infection or trauma by increasing the susceptibility to pain after exposure to a trigger (for example infection) and/or by decreasing the ability to terminate the inflammatory process (Gerber et al., 2003). The interleukin-1beta (IL-1β) gene (Gerber et al., 2003), the interleukin-1 receptor antagonist gene (Jeremias et al., 2000) and the mannose-binding lectin (MBL) gene (Babula et al., 2004), at least, have been studied in association with LPV.

2.2.5.5 Musculoskeletal system

Vaginal delivery, trauma, pelvic or abdominal surgery can change the biomechanics and physiology of the pelvic-floor musculature (Pukall et al., 2016). Increased pelvic-floor muscle tone and reduced stretching capability have been found in LPV patients (Morin et al., 2014; Morin et al., 2017; Thibault-Gagnon et al., 2015). Women with LPV also showed decreased strength, coordination, endurance and speed of contraction of the pelvic-floor muscles compared with healthy controls (Morin et al., 2017). A growing body of evidence acknowledges pelvic-floor muscle hypertonicity in the pathophysiology of LPV, yet the mechanisms are still unclear (Thibault-Gagnon et al., 2015). In addition to pathophysiology, pelvic-floor hypertonicity can contribute to pain sensation because pain may trigger a reflex contraction and increased tension in the pelvic-floor muscles in LPV patients (Gentilcore-Saulnier et al., 2010).

2.2.5.6 Central and peripheral sensitization of the nervous system

The experience of pain is a complex process that activates multiple neuronal signaling pathways in the central nervous system (CNS) and peripheral nervous system (PNS). The experience of pain can be summarized as a four-stage process: transduction, transmission, modulation and perception (Dureja et al., 2017). In the transduction stage I, nociceptive stimuli of tissue-damaging potential (mechanical, chemical or thermal) are converted by the sensory cells into action potentials. Stage II, transmission, involves the conduction of these action potentials via afferent neurons to the dorsal horn of the spinal cord. In the modulation stage (III) coding of nociceptive information occurs at the level of the spinal dorsal horn. The

modulation process at the dorsal horn can be excitatory or inhibitory, thereby decreasing or increasing the resulting pain. In stage IV, pain perception is generated by autonomic, affective, cognitive and behavioral responses to the painful stimulus (Dureja et al., 2017).

In peripheral sensitization, afferent nociceptors (A-delta and C-type) develop abnormal sensitivity to noxious stimuli. A-delta fibers are myelinated and transmit painful stimuli fast, whereas C-type fibers are nonmyelinated and are responsible for the slow transmission of pain (Rocha et al., 2007). Nociceptors of the skin and deeper tissues may become extremely sensitive to noxious stimuli in the presence of inflammation. This lowers the threshold of nociceptor activation to normally less-painful stimuli and at the same time the degree of response is increased (Dureja et al., 2017). Also, extreme sensitization may lead to the activation of silent nociceptors, which upon excitation amplify the pain response manifold. Drugs targeting peripheral nociceptors include nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, cannabinoids and transient-receptor potential vanilloid (TRPV1) receptor antagonists (Dureja et al., 2017).

Central sensitization implies changes in peripheral impulses, with positive or negative adaptations. A reduction in the threshold or an increase in the response to afferent impulses occurs as along with persistent discharges of neuroactive substances after repeated stimuli and widening of the receptive fields of the dorsal-horn neurons (Rocha et al., 2007). This phenomenon of altered sensitivity of neuronal cells at the level of the CNS is known as central sensitization. The central sensitization process transits acute pain to chronic pain. Primary hyperalgesia (Table 5) is the first manifestation of altered threshold at the central neuronal level. Hyperalgesia can be divided into primary and secondary hyperalgesia. Primary hyperalgesia means an increased response to a painful stimulus at the site of injury, and secondary hyperalgesia is the extension of that response to adjacent areas (Rocha et al., 2007). Under pathological conditions, receptors that are normally associated with sensory responses to stimuli such as touch, may gain the ability to produce pain. This results in secondary hyperalgesia, an important aspect of central sensitization. In contrast to peripheral nociception, a number of neurochemical drivers modify pain perception at the central level, which creates the complex interplay of events that underlie the pathology of many chronic and neuropathic pain conditions (Latremoliere et al., 2009).

Table 5. Terminology of pain

Allodynia	A nonpainful stimulus felt as painful in spite of normal appearing tissues
Dysestesia	Unpleasant abnormal sensation, spontaneous or provoked
Hyperalgesia	Increased sensitivity to painful stimulus
Hyperestesia	Increased sensitivity to stimulus
Hyperpatia	Increased response to repetitive, nonpainful stimulus
Hypoalgesia	Decreased sensation to painful stimulus
Hypoestesia	Decresed sensitivity to stimulus

Central and peripheral sensitization may be responsible for the pain symptoms experienced long after any triggering factor has been resolved (Danby et al., 2010). Reduced pressure-pain and sensory thresholds (Pukall et al., 2002) as well as altered central sensitization (Zhang et al., 2011) have been reported in women with LPV.

2.2.5.7 Psychological factors

The significance of psychological factors in the development of vulvodynia has been relatively well studied. Associations between depression (Iglesias-Rios et al., 2015; Khandker et al., 2011; Nylanderlundqvist et al., 2003), posttraumatic stress disorder (PTSD) (Iglesias-Rios et al., 2015), anxiety (Khandker et al., 2011; Nylanderlundqvist et al., 2003) somatization (Granot et al., 2005) and lower-body image (Granot et al., 2005) with vulvodynia have been found in several studies. However, the question whether psychological comorbidities are involved in the pathophysiology of vulvodynia, are associated with vulvodynia or worsen because of vulvodynia remains unanswered. It is also possible that psychological factors affect various aspects of vulvodynia. For example, when studying depression and chronic pain, it has been hypothesized that the impact is most likely bi-directional: depression may contribute to pain development and increased sensitivity to pain (Miller et al., 2018). In addition, a patient with a chronic pain condition may withdraw from pleasurable activities, and increased helplessness and distress that accompany chronic pain can further worsen the symptoms of depression (Miller et al., 2018).

2.2.5.8 Other pain syndromes associated with LPV

In a study by Reed et al. 45 % of 1847 women with vulvodynia reported having at least one of five chronic pain conditions: chronic fatigue syndrome, endometriosis, fibromyalgia, interstitial cystitis or irritable bowel syndrome (Reed, 2012$_b$). In a

population based study of over 1900 women, patients with vulvodynia had more frequently interstitial cystitis, fibromyalgia and irritable bowel syndrome (Reed, 2012b).

2.2.6 Quality of life and sexuality of vulvodynia patients

Women suffering from vulvodynia report a worse overall quality of life (QoL) than women with no vulvodynia (Arnold et al., 2006). Moreover, over 40 % of patients with vulvodynia felt out of control with their life and 60% felt out of control with their body as a consequence of vulvodynia (Arnold et al., 2006). Vulvovaginal pain has a negative impact on woman's sexuality and intimate relationships (Bergeron et al., 2014). In a qualitative study of 33 vulvodynia patients, both woman's and partner's fear of genital pain during intercourse prevented the pleasure of sexual act even more than the actual pain (Törnävä, 2017). Some patients had experienced pressure for sexual act in their relationship and the responsibility to please the partner even if they didn't feel comfortable about it themselves (Törnävä, 2017).

2.2.7 Treatment of localized provoked vulvodynia

Only a few randomized controlled studies on vulvodynia treatments are available. Most of the studies regarding treatment options are based on prospective or retrospective designs, or case reports, and typically lack a control group. Furthermore, most of the studies have enrolled only LPV-patients. Despite the fact that relatively few good-quality studies have been conducted, different treatment modalities can still relieve vulvodynia-related pain sufficiently in most cases.

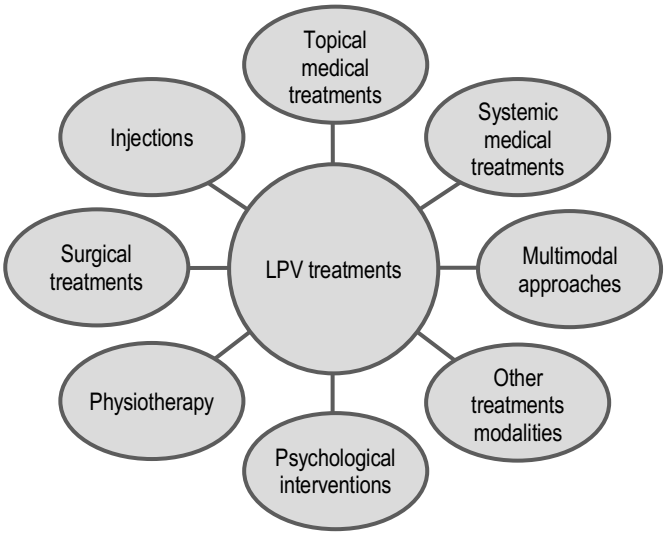

Figure 4. LPV treatments

2.2.7.1 Topical medical treatments

Topical medical treatments lack typical side-effects of systemic medication and therefore are usually better tolerated. A summary of topical medical treatments for LPV is presented in Table 6.

Table 6. Summary of topical medical treatments used in LPV

Topical treatment	Study	Materials & Methods	Results
Lidocaine 5%	Foster, 2010	112 pts with LPV, randomized, controlled study. Lidocaine only, lidocaine+ oral desipramine, placebo for 12 weeks	Lidocaine only or combined to oral desipramine not better than the placebo
Lidocaine 2% and 5%	Danielsson, 2006	46 pts with LPV randomized, prospective study. Lidocaine vs EMG biofeedback for four months	Both groups improved equally
Lidocaine 5%	Zolnoun, 2003	61 pts with LPV, prospective study, lidocaine for seven weeks	76% of pts were able to have intercourse (36% before treatment) p=0.002
Topical gabapentin 2-6%	Boardman, 2008	51 pts with LPV or GUV, minimum eight weeks	Pain improved more than 50% in both groups
Estradiol 0.03% and testosterone 0.01%	Burrows, 2013	50 pts with LPV while using CHC, cessation of CHC and started topical treatment	Significant improvement in vestibular pain scores (p=0.001)
Amitriptyline 2%	Pagano, 2012	Prospective study of 150 pts: 102 pts with LPV, 48 with localized provoked/unprovoked vulvodynia	56 % responded to treatment, 15 pts completely pain free, 69 pts slight or moderate response
Amitriptyline 2%/ baclofen 2%	Nyirjesy, 2009	Retrospective study, 38 pts with LPV	Symptom relieve in 71% of pts, 29% reported no or little improvement
Cromolyn cream 4%	Nyirjesy, 2001	26 LPV pts in randomized controlled study, cromolyn vs placebo for three months	Both groups improved equally
Nifedipine 2%-4%	Bornstein, 2010	Randomized placebo-controlled study of 30 pts with LPV, 10 pts placebo, 10 pts nifedipine 2%, 10 pts nifedipine 4% for six weeks	No differences between groups
Nitroglycerine 0.2%	Walsh, 2002	34 pts with vulvodynia, a prospective study	91% pts reported improved pain
Capsaicin 0.05%	Murina, 2018	33 pts with LPV treated with capsaicin for six months by decreasing doses, a prospective study	59% of pts improved but symptoms recurred after cessation of capsaicin cream
Cutaneous fetal fibroblast lysate	Donders, 2012	26 LPV pts in randomized controlled study, cromolyn vs placebo for three months	Modest but significant improvement in pain and focal redness compared to placebo
Vaginal diazepam	Murina, 2018	Randomized, placebo-controlled study 21 LPV ptswith diazepam+TENS, 21 pts placebo+TENS	Both groups improved equally
1% Hydrocortison cream	Bergeron, 2016	97 pts with LPV randomized into topical steroid group and gCBT	Both groups improved significantly in pain, gCBT group improved significantly better at 6 mo's follow-up

Pts Patients
EMG Electromyography
CHC Combined hormonal contraceptives
gCBT Group cognitive behavioral therapy
TENS Transcutaneous nerve stimulation

In a review of vulvodynia assessment and treatment (Goldstein et al., 2016), topical lidocaine is not recommended as a long-term treatment for LPV (grade B). Likewise capsaicin is not recommended as a first-line treatment, but it can be considered if other treatments fail or as an alternative to surgery (grade C) (Goldstein et al., 2016). Topical corticosteroids are not recommended for LPV (grade C) (Goldstein et al., 2016). As a summary, there is no first line recommendation on any

of the topical medical treatments (Goldstein et al., 2016), however, side effects are rare.

2.2.7.2 Systemic medical treatments

The two most widely used forms of systemic medications for vulvodynia are the anticonvulsants and antidepressants. A summary of systemic medical treatments in shown in Table 7.

Table 7. Anticonvulsants and antidepressants and a in the treatment of vulvodynia

Medication	Study	Materials & Methods	Results/Conclusions
Gabapentin (Lamotrigine in one study)	Review. Spoelstra, 2013	8 studies, 327 pts with GUV and LPV (1 non-randomized prospective study, 2 retrospective studies, 1 retrospective review, 1 open label pilot study, 3 case reports)	Due to several methodological weaknesses, more good quality studies are needed. The evidence is insufficient for recommendation.
Gabapentin 1200-3000 mg/day	Brown, 2018	Multicenter, double-blind, randomized, placebo-controlled crossover trial, 89 LPV pts (45 gabapentin, 44 placebo)	Gabapentin did not reduce the tampon-test pain, however sexual function improved in pts with LPV and increased pelvic muscle pain (Bachmann 2019)
Tri-cyclic antidepressants (mainly amitriptyline), SSRI (two studies), SNRI (one study)	Review. Leo, 2013,	13 studies, 787 pts with LPV or GUV (2 RCT's, 1 randomized open-label trial, 4 prospective studies, 3 retrospective reviews, 3 case reports	Insufficient evidence to support the use of antidepressants for vulvodynia, more studies needed
SSRI (escitalopram) in combination with perfenazine and TCA (amitriptyline)	Tribo, 2008	Prospective study, 80 (55 received treatment, 25 controls) pts with LPV or GUV, 6 months of treatment	52.7% complete remission of symptoms in the treatment arm, 12 % remission in control arm
SNRI (milnacipran) 50-200 mg/day	Brown, 2015	Open-label trial, 22 LPV pts received medication for 12 weeks	Significantly reduced vestibular pain

Pts = Patients
SSRI = Selective serotonin reuptake inhibitor
SNRI = Serotonin and norepinephrine reuptake inhibitor
RCT = Randomized controlled trial
TCA = Tri-cyclic antidepressant

Tricyclic antidepressants (TCA) have proven their efficacy in the treatment of neuropathic pain (Dobecki et al., 2006). However, according to a systematic review covering 13 reports the evidence supporting the use of antidepressant pharmacotherapy in the treatment of vulvodynia was insufficient (Leo et al., 2013),

and mostly based on this review, Goldstein et al. (2016) recommended against TCA treatment in LPV (Grade A). A few small studies have addressed the use of selective serotonin reuptake inhibitor (SSRI)) antidepressants in combination with amitriptyline and perfenazine (Tribo et al., 2008), or serotonin and norepinephrine reuptake inhibitor (SNRI) antidepressant (milnacipran) (Brown et al., 2015) in the treatment of vulvodynia, with preliminary good results but more research is warranted. Anticonvulsants are not recommended for LPV pain reduction, however, sexual functioning may improve in women with LPV and increased pelvic muscle pain (Bachmann et al., 2019)

2.2.7.3 Injections

In clinical practice submucosal injections of anti-inflammatory corticosteroids combined with lidocaine have been used, although their efficacy has not verified in RCTs. However, in a small series of 22 women with LPV, with methylprednisolone and lidocaine at decreasing doses (1.0 ml, 0.5 ml and 0.3 ml) injected in the vulvar vestibule, in 68% of the patients improved and nearly half of them were asymptomatic after nine months (Murina et al., 2001). Similar results regarding bethametasone and lidocaine injections six times in six weeks have also been reported in few case reports (n=1) (Dede et al., 2006; Segal et al., 2003).

Of 20 women with LPV (Friedrich's criteria fulfilled) and histopathologically confirmed human papillomavirus (HPV) infection (typical koilocytotic atypia), seven patients were treated with interferon alpha injections (three times a week, 106 units interferon α-2b intradermally). Four of the women were completely cured and three achieved partial resolution of symptoms (Sonnendecker et al., 1993). However, interferon treatment alone lacks evidence of efficacy in a RCT setting and therefore is not recommended as first-line treatment for LPV (Goldstein et al., 2016).

Heparanase is excreted by mast-cells; it directly activates endothelial cells and elicits angiogenic responses (Farajun et al., 2012). It is suggested that heparanase may play a role in degradation of the vestibular stroma, allowing nerve fibers to penetrate the epithelial basement membrane. This may lead to hyperinnervation in the vestibular area which may be the cause of hypersensitivity to touch in women with LPV (Farajun et al., 2012). Heparin and enoxaparin have been found to be inhibitors of heparanase activity (Farajun et al., 2012). In the RCT of LPV with 40 mg enoxaparin subcutaneously for 90 days, 15 out of 20 women reported over 20% pain reduction compared with five out of 18 in the placebo group. The injections can cause bruising and bleeding and may therefore be an inconvenient treatment option

for some patients (Farajun et al., 2012). The authors concluded that enoxaparin reduced the number of intraepithelial free nerve fibers in women with vulvodynia.

The hypothesized mechanism of botulinum toxin (BT) action in vulvodynia patients is that it may inhibit neurotransmitters associated with inflammation and pain and also reduce the pelvic-floor muscle spasms observed in many patients (Falsetta et al., 2017). In one study (n=33), BT type A injected subcutaneously to the vestibular area (doses of 100 U and 50 U) did not perform better than placebo after three months of follow-up, but pain reduction was found in all the treatment groups (Diomande et al., 2019). Repeated injections of BT type A (100 U) reduced pain and also increased the pain threshold level, but this second part of the study did not include any placebo injections (Diomande et al., 2019). Another RCT with LPV patients did not prove Botulinum toxin type A (20 U, n=32) to be superior to placebo (n=32) after three and six months of follow-up, but again both groups reported relief in pain (Petersen et al., 2009). Non-controlled studies have shown efficacy of BT type A, but in a systematic review by Goldstein et al. (2016) BT-A was suggested to be used only as a second-line treatment.

2.2.7.4 Physiotherapy

Pelvic-floor muscle physiotherapy is recommended as a first-line treatment for LPV in clinical guidelines (Goldstein et al., 2016; Stockdale et al., 2014). Physiotherapy for LPV consists of various treatment modalities, such as biofeedback, which is aimed at gaining better control of the pelvic-floor musculature, promoting muscle relaxation and improving contractile properties (Moyal-Barracco et al., 2010). In a systematic review covering 43 studies on physiotherapy for LPV (n=1332 women) biofeedback, dilators, electrical stimulation, education, multimodal physical therapy and multidisciplinary therapies were found to be effective for decreasing coital pain and improving sexual function, although more well-designed research was warranted to assess the efficacy (Morin et al., 2017). A randomized prospective study on electromyographic (EMG) biofeedback vs. topical lidocaine showed improvement in both groups regarding pain, sexual functioning and psychosocial adjustment at the 12-month follow-up, with no differences between these two groups. In a randomized study, superficial electromyographic (sEMG,n=28) biofeedback, vestibulectomy (n=22) and cognitive behavioral therapy (CBT, n=26) were equally good treatment modalities regarding positive sexual function and psychological adjustment outcomes, but vestibulectomy was more successful in reducing vestibular pain (Bergeron et al., 2001). Instead, in a RCT active (n=20) and sham (n=20)

transcranial direct-current stimulation for LPV did not differ in terms of pain, sexual function, vestibular sensitivity or psychological distress (Morin et al., 2017). In another RCT, including 40 women with LPV, transcutaneous electrical nerve stimulation (TENS, n=20) showed improvement in pain compared with placebo (n=20) (Murina et al., 2008). In a prospective study of 18 women with superficial dyspareunia, vaginal dilators relieved the symptoms in almost 77.8% of the patients (Idama et al., 2000).

2.2.7.5 Psychological interventions

Psychological interventions for LPV are targeted at reducing the pain, improving sexual functioning and strengthening the couple's relationship. Sexual pain affects thoughts, behavior, emotions and a couple's interaction. Psychological interventions can be carried out in a group, or on an individual or couple-based level. Cognitive behavioral therapy (CBT) is probably the most studied and used therapy for chronic pain including vulvar and sexual pain. It is described as a tool for coping with the negative thoughts induced by chronic pain (Ledger et al., 2014). In one of the first RCT, which compared group CBT, biofeedback (n=28) and vestibulectomy (n=22), group CBT (n=26) resulted in similar improvement in sexual function and psychological adjustment compared to the other treatments. However, vestibulectomy was superior in pain reduction (Bergeron et al., 2001). The benefits of CBT were retained up to a 2.5-year follow-up point (Bergeron et al., 2001). When CBT was compared to supportive psychotherapy (42 participants with LPV) in a RCT, CBT was found to reduce pain and improve sexual function better than supportive, non-directional talk-therapy and the results were maintained at one-year follow-up (Masheb et al., 2009).

Mindfulness-based therapies (MBTs) have been shown to be efficacious in treating various conditions such as pain, depression and anxiety (Lakhan et al., 2013). Mindfulness techniques are thought to encourage acceptance and non-judgement, which may be helpful in lessening LPV-related symptoms (Dunkley et al., 2016). The potential aid of mindfulness has led researchers to develop and test mindfulness-based CBT (MCBT) for sexual pain and LPV. Mindfulness-based group cognitive therapy (M-gCBT) (n=14) was compared with education support group (n=17) therapy for LPV in a RCT (Guillet et al., 2019). Both groups improved in terms of pain (tampon test) and the two methods were considered equally good in pain control. Women in the M-gCBT group improved more in sexual functioning, with reduction in anxiety and depression (Guillet et al., 2019). In a review, psychological

interventions were recommended as treatment for vulvodynia (grade-B evidence) (Goldstein et al., 2016).

2.2.7.6 Multimodal approaches

A 10-week hospital-based multidisciplinary vulvodynia program (MVP), which included psychological therapy, pelvic-floor physiotherapy and medical management of LPV patients (n=132) yielded improvements in dyspareunia, sex-related distress and sexual functioning (Brotto et al., 2015). Improvements were retained at three and four months of follow-up (Brotto et al., 2015), but longer-term results were not collected in that study (Brotto et al., 2015). A multidimensional approach to LPV resulted in remarkable pain reduction in 81% of a study group (n=64) and 80% of the women had resumed intercourse (Spoelstra et al., 2011). In a RCT of LPV patients (n = 14) a "behavioral approach" was compared with that alone or combined with vestibulectomy (Weijmar Schultz et al., 1996). The behavioral approach consisted of patient education, biofeedback, pelvic-floor muscle exercises, hygienic protocol, sexual counseling and sexological-partner-relation therapy if appropriate. In the second non-randomized part of the study, women and their partners were given the opportunity to choose whether or not to include surgery. Of all women, 82% chose behavioral treatment only. Two to three years after treatment, 79% of the patients in RCT part of the study and 89% of the patients of the non-randomized part of the study reported improvement. The outcomes did not differ in the surgical and non-surgical group (Weijmar Schultz et al., 1996). Interdisciplinary treatment is recommended in the management of vulvodynia (grade C), but further studies are warranted to evaluate the efficacy of this treatment (Goldstein et al., 2016).

2.2.7.7 Surgical treatments

Surgery for LPV is generally considered as the last treatment modality and should be used only when conservative treatments fail, albeit the efficacy of surgery is well established in different studies (Goldstein et al., 2016). Surgery is not recommended for patients with GUV or patients whose pain is not provoked (Tommola et al., 2010). Surgical treatment success rates vary between 61% and 94% (Landry et al., 2008). A summarization of different techniques used in studies is described in Table 8.

Table 8. Studies on different surgical techniques for vulvodynia treatment

Procedure	Study	Materials & Methods	Results
Perineoplasty, excision of a semicircular segment of perineal skin, the posterior hymeneal ring and the mucosa of the posterior vulvar vestibule	Woodruff, 1981	A case series of 42 pts, mainly suffering from dyspareunia	Dyspareunia was alleviated in all cases, 6 mos-5 yrs after surgery
Vestibulectomy, excision is limited to sensitive areas in the vestibulum and hymen, an u-shaped incision to the depth of 2 mm	Bergeron, 1997	Retrospective study of 38 LPV pts	63.2 % of pts yielded a positive outcome, 36.8% moderate or no improvement
Modified vestibulectomy, excision is continued up to the parameatal region superiorly to Hart's line and inferiorly following Hart's line, vestibular mucosa excised past the hymeneal ring	Goldstein, 2006	Retrospective cohort study, 134 LPV pts	93 % were satisfied with the outcome after mean 26 mos of follow-up
Modified posterior vestibulectomy, removal of mucosa of the posterior vestibule only	Tommola, 2011	Retrospective cohort study, 70 LPV pts	57 out of 70 pts attended the follow up visit after surgery (median 36 mos after surgery), VAS for dyspareunia decreased median 9-->3, 91 % were satisfied with the outcome
A simplified surgical technique for vulvodynia, excision on all tender parts of the vestibulum, skinning technique, major part of hymen left intact, no need for vaginal advancement	Goetsch, 1996	A feasibility study, 12 LPV pts	10 out of 12 pts had complete resolution of symptoms 6 mos-6 yrs after surgery
Vestibuloplasty, incision made to anterior, parameatal and posterior vestibular mucosa which is the undermined and resutured, no tissue removal	Bornstein, 1995	21 LPV pts randomized to vestibuloplasty or perineoplasty	9 out of 11 pts complete remission in perineoplasty group, none of 10 pts in vestibuloplasty group resolved after 6 weeks-6 mos of operation

There is a significant variation in surgical techniques used, different surgeries are characterized by the same name in different studies (Goldstein et al., 2016). At least perineoplasty (Woodruff et al., 1981), vestibulectomy (Bergeron et al., 1997), modified posterior vestibulectomy (Tommola et al., 2011), simplified surgical technique (Goetsch, 1996) and vestibuloplasty (Bornstein et al., 1995). The variety of different techniques, terms describing surgical procedures and definitions of successful outcome makes difficult to compare the results (Goldstein et al., 2016). In a systematic review of 33 studies on surgical treatment the authors concluded that

surgery seemed to be effective and there is no single superior surgical technique for treating LPV (Tommola et al., 2010).

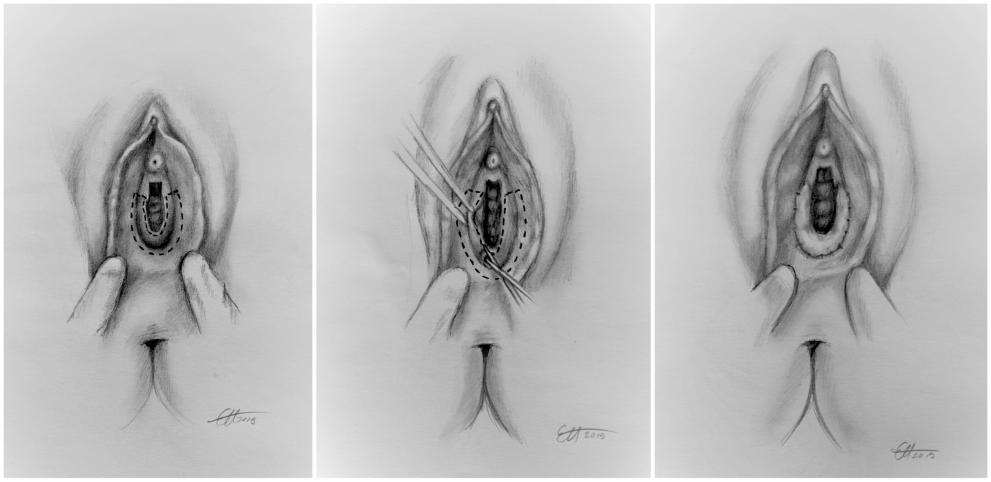

Figure 5. Modified posterior vestibulectomy

In one of the few published RCT's, vestibulectomy was more effective in pain reduction than sEMG biofeedback or CBT (Bergeron et al., 2001) at the post-treatment visit and after six months of follow-up. However, the results in that study have to be interpreted with caution because several patients randomized to the vestibulectomy group chose another treatment (sEMG biofeedback, CBT) instead. Sexual and psychological functions improved equally in all three groups (Bergeron et al., 2001). Another study that included randomization to surgical (behavioral treatment and surgery) and nonsurgical (behavioral treatment only) groups, showed no difference between these two treatment modalities after 2.5–3 years of follow-up (Weijmar Schultz et al., 1996). The surgical procedure used in that study was modified Woodruff perineoplasty (Weijmar Schultz et al., 1996).

Tommola et al. reported that posterior vestibulectomy and conservative treatment both decrease dyspareunia, and long-term sexual well-being was similar in both treatment groups (Tommola et al., 2012). In another study, after a median follow-up time of 41 months, vestibulectomy had reduced coital pain VAS scores from eight to two and major improvement was reported by 56% of women with secondary LPV (Bohm-Starke et al., 2008). A total of 79% of operated women reported improved psychological well-being (Bohm-Starke et al., 2008). Vestibulectomy is currently suggested as a treatment option only if other less-

invasive treatment options have been attempted (grade B evidence) (Goldstein et al., 2016).

2.2.7.8 Other treatment modalities

Low-level laser therapy (LLLT) has been successfully used in chronic pain conditions (Lev-Sagie et al., 2017), although the exact mechanism of laser treatment in pain relief is unknown. However, in a RCT, LLLT,n = 18) was not superior to placebo (n = 16) among LPV patients (Lev-Sagie et al., 2017). In a consensus document, based on the few studies published on laser treatment for vulvodynia (Leclair et al., 2007; Lev-Sagie et al., 2017; Murina et al., 2016), LLLT is not recommended for vulvodynia (Preti et al., 2019).

Acupuncture treatment, on the basis of one RCT of 36 women with LPV, decreased pain and improved sexual function compared with the waiting-list controls who did not receive treatment (Schlaeger et al., 2015).

2.2.7.9 The placebo effect

In a systematic review of RCTs on vulvodynia medication, including 297 LPV patients, no advantage of any medication over placebo was found. In some cases, the placebo was even more efficient than the actual drug (Miranda Varella Pereira et al., 2018).

2.2.8 Cost of vulvodynia and other chronic pelvic pain conditions to society

Vulvodynia is known to cause significant physical and psychological distress and worsening of QoL for the affected women (Arnold et al., 2006). Like in other chronic pain conditions, the cost to society may be high if the diagnosis and proper treatment are delayed. Arnold et al. reported that most women suffering from vulvodynia (75%) had consulted three to nine doctors in their lifetime for their vulvar pain condition, and one quarter had missed work at least once in the previous year due to symptoms of vulvodynia (Arnold et al., 2006). In another study, during a 12-month follow-up period, vulvodynia patients (n = 12,584) had a mean of 20.9 outpatient clinic visits and 0.5 emergency department visits (Lua et al., 2017). As many as 8.2%

of the study population had at least one period of inpatient hospitalization (Lua et al., 2017).

Few investigators have attempted to estimate the economic burden of vulvodynia. Xie et al. (Xie et al., 2012) found that the annual costs in the US range from 31–72 billion dollars. The total cost in six months per patient was 8862.40 dollars, which included direct healthcare costs (insurance, medication etc), direct non-healthcare costs (transportation) and indirect costs (sick leave, loss of employment, employee costs, need for assistance in household work etc.) (Xie et al., 2012).

3 AIMS OF THE STUDY

The studies in this thesis were designed to investigate etiological factors, different treatment methods and clinical outcomes of vulvodynia patients. Specific aims of the study were as follows:

1. To assess the characteristics, treatments and treatment outcomes of a vulvodynia patients.
2. To assess the clinical outcomes and QoL related to surgical and non-surgical LPV treatments. QoL data of LPV patients was also compared with population-based QoL data of healthy age-matched women.
3. To analyze the immunohistochemical expression of hormonal and inflammatory factors in vulvar tissue derived from LPV patients and healthy controls.
4. To analyze the vulvar microbiome of LPV patients compared to healthy controls using the next generation sequencing (NGS).

4 SUBJECTS AND METHODS

4.1 Subjects and study design

Studies I and II were single-center retrospective patient-cohort studies on vulvodynia patients treated in TAUH from 2003 to 2013 (Study I) and from 2003 to May 2016 (Study II). Study III was a retrospective patient-cohort study of vestibulectomy patients treated in TAUH in 2003–May 2016. Study IV was a prospective patient-cohort study of LPV patients. The controls for Studies III and IV were recruited prospectively from TAUH and Kanta-Häme Central Hospital from 2018 to 2019 among patients who were treated for menstrual disorders.

4.2 Methods

4.2.1 Collection of retrospective patient cohort (Study I-II)

The data for the retrospective patient-cohort studies were collected from electronic medical records of TAUH. To identify the vulvodynia patients for Study I, the following ICD codes were used for patients treated in TAUH in 2003–2013: N94.1 Dyspareunia, N94.2 Vaginismus, N90.8 Other specified noninflammatory diseases of vulva and perineum and N90.9 Noninflammatory disease of vulva and perineum, unspecified. Patient records were read through and those who were at least 18 years old, fulfilled Friedrich's two criteria (pain on attempted vaginal entry and tenderness to pressure localized within the vulvar vestibule, (Friedrich, 1987)) and had symptoms that had lasted for a minimum of three months were included. Patients diagnosed with candidiasis or other infections of the vagina and/or vulva, with resolving symptoms after appropriate treatment, were excluded from the study. Patients were also excluded if the pain was related to some detectable benign or malignant diseases of the vulva. For 133 vulvodynia patients identified in the medical records, clinical data, treatments, number of outpatient visits and the patients' demographic factors were collected. The patient flow chart is described in Figure 6.

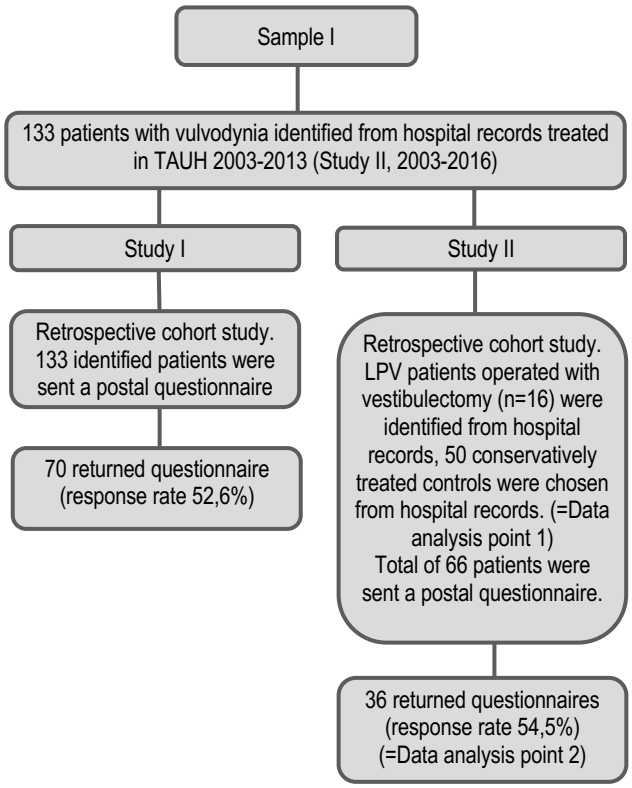

Figure 6. Patient flow chart (Study I – II)

To identify the LPV patients treated by means of vestibulectomy in 2003–May 2016 (Study II) another search was conducted using the same ICD codes as in Study I and identified the patients with LPV according to Friedrich's criteria (Figure 6). All patients that had undergone vestibulectomy were identified from the medical records and included in Study II (n = 16). To enroll non-surgically treated LPV controls to the study, 50 successive LPV patients fulfilling Friedrich's criteria were identified from the patient records (triple the amount of surgically treated patients). The same exclusion criteria were used as in Study I. All the demographic and clinical data were collected from the hospital records at data analysis point 1, two months after commencing the treatments (Figure 6). In addition to compare the two study groups (Study II) in terms of QoL, a group of healthy women aged 25–34 from the Finnish national register was identified for comparison.

4.2.2 Collection of prospective patient cohort (Study III & IV)

LPV patients that had been treated by means of vestibulectomy (Study III) were the same as identified for Study II. Vestibulectomy samples for Study III were collected from the hospital archives (all vestibulectomies performed at TAUH in 2003– May 2016) (Figure 7). Patients with vulvar malignancy, or ongoing inflammatory or skin diseases of the vulva were excluded. LPV patients for Study IV were recruited in the gynecological outpatient clinics at TAUH and Kanta-Häme Central hospital from January 2018 to March 2019 (Figure 7). The patients that fulfilled Friedrich's first two criteria for LPV (Friedrich, 1987) and came for the first visit or a routine follow-up visit to one of the outpatient clinics were recruited to this study.

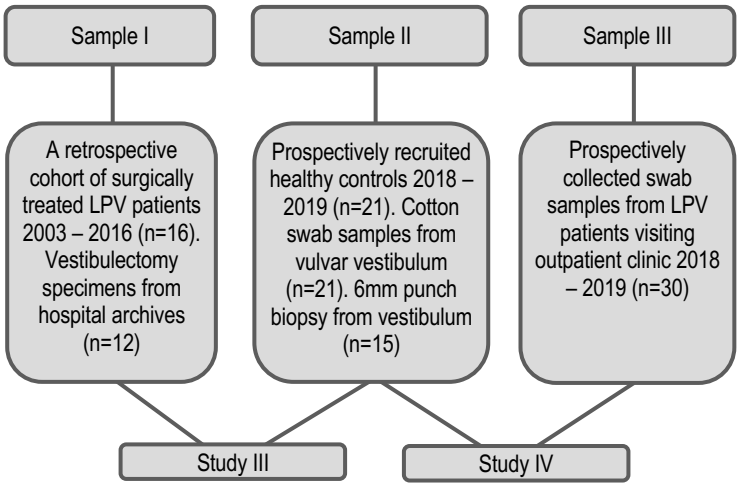

Figure 7. Patient flow chart (Study III and IV)

Controls (Studies III and IV) were prospectively recruited among volunteers aged 18–40, admitted to hysteroscopy for benign reasons (generally hypermenorrhea with a polyp or a fibroid) from TAUH and Kanta-Häme Central Hospital from January 2018 to March 2019. The exclusion criteria were: pregnancy, history of vulvar malignancy, any inflammatory or skin disease of any part of the body and any type of localized or generalized pain syndrome.

Demographic data (age, parity, menopausal status, treatments given before vestibulectomy, medication) on LPV patients were collected from hospital registries. For controls, a short questionnaire concerning demographic data, current

medication, and phase of the menstrual cycle was filled by a physician at the time of the punch biopsy.

4.2.3 Study questionnaires (Study I-II)

In Studies I and II a postal questionnaire was sent to the identified vulvodynia patients. In Study I the participants were asked to assess the treatment, QoL and treatment satisfaction. A numerical rating scale (NRS; 0–10) was used to quantify the intensity of vulvar pain, where 0 was "no pain" and 10 was "the worst imaginable pain". Questionnaires were re-sent to patients who did not return them within 1.5 months after the first mailing. A detailed description of the questionnaire is presented in Table 9. Study participants were also asked to rate their experiences with treatments given by various professionals, scores of 4 and 5 was considered satisfactory. QoL was measured using a 0-5 scale and it was considered good if a patient reported scores of 4 ("satisfied") or 5 ("very satisfied").

Table 9. Content of the questionnaire sent to vulvodynia patients in Study I

Backround Information
Age
Nulliparous/number of births
Symptoms before/after giving birth
Recurrent yeast infections yes/no, diagnosed by a physician yes/no
Symptoms before/after yeast infections
Bacterial vaginosis yes/no, diagnosed by a physician yes/no
Symptoms before/after bacterial vaginosis
Hormonal contraception yes/no, name of the contraceptives used
Other medications (name of the medication)
Beginning of symptoms, at which age
Delay between first symptoms and treatment
First contact about the symptoms (e.g. Public health center/private doctor)
Unit that referred patient to University Hospital (e.g. Public health center/Private)?
Name all treatment modalities you received for vulvodynia (examples given)
Pain (NRS scale, 0= no pain, 10= worst pain imaginable)
Pain before treatments
Pain after treatments
Vulvodynia symptoms
Are the symptoms local/generalized?
Provoked/not
Patient satisfaction (5-point scale, 0=Not satisfied at all, 5=Very satisfied)
Referral to University hospital, on time
Treatment protocol satisfaction
Information given about vulvodynia verbal/written
Satisfaction to the physician
Satisfaction to the sexual counselling by a trained nurse
Satisfaction to the physiotherapist
Efficacy of different treatments received
Quality of life
Partner satisfaction
Relationship satisfaction after treatments

In study II a seven-page postal questionnaire on demographic data, self-reported pain measured with NRS and validated QoL-questionnaire RAND-36 was sent to eligible patients. The validated Finnish version of the RAND-36-item health survey includes eight multi-item dimensions: general health, physical functioning, mental health, social functioning, vitality, pain, and physical and emotional role functioning ("36-Item Short Form Survey Instrument (SF-36)/RAND,"; Aalto A-M, Aro AR, 1999). During RAND-36 validation process in Finland, a study sample of 1,038 women was collected from Finland's national register. QoL was measured in different age groups among Finnish women (Aalto A-M, Aro AR, 1999). The returned questionnaires were analyzed at data analysis point 2.

4.2.4 Vulval clinical sample collection (Study III-IV)

Two cotton-swab samples were taken from the vulvar vestibulum during the gynecological examination from the LPV patients (Figure 7). Samples were stored at -70 degrees in the laboratory for further analysis by immunohistochemistry and bacterial DNA sequencing. The Control group consisted of women who were admitted to hysteroscopy for benign reasons (generally hypermenorrhea with a polyp or a fibroid) under general anesthesia or as an office procedure. Before the procedure, two cotton-swab samples were taken from the vulvar vestibulum during the gynecological examination and stored at -70 degrees in the laboratory for further analysis by immunohistochemistry and bacterial DNA sequencing. For the punch biopsy, local anesthetic agents (1–2 ml of 0.01% lidocaine with adrenaline) were used in cases of outpatient clinic hysteroscopy and a 6-mm punch biopsy sample from the vulvar vestibulum at 7 o'clock was taken. Punch biopsy samples were routinely embedded in paraffin after a maximum of 24 hours of fixation in 10% buffered formalin. All control biopsies were taken at a standardized time point of the menstrual cycle (before cycle day 12).

4.2.5 Immunohistochemistry (Study III)

All vestibulectomy specimens (modified posterior vestibulectomies performed in TAUH between January 2003 and May 2016) were collected from the hospital archives (n = 12). Although the original number of identified patients treated by means of vestibulectomy was 16, all the samples were not found from the archives for the following reasons: one patient was operated upon in the USA, one at Helsinki University Central Hospital (HUCH), one in a district hospital in TAUH and the sample was not in the archives, and in one patient the sample was missing for an unknown reason. All patients operated upon had been diagnosed with LPV before surgery. The macroscopic and morphological findings concerning the vestibulectomy specimens were confirmed by an experienced pathologist (RH-O) as a part of routine diagnostics in The Department of Pathology at Fimlab Laboratories Ltd. The precise methods for preparing the IHC samples are described in Study III.

All stainings were evaluated by an experienced dermatopathologist (RH-O) and gynecologist (AA). Immunohistological sections were analyzed under a light microscope (Olympus BX51, Model U-MDOB3, Tokyo, Japan) from representative areas. Staining patterns of ERs and PRs were scored in a manner similar to that used

in routine breast pathology on a 0–3 scale: 0 = negative; 1 = less than 10%; 2 = 11–50%; 3 = 51–100% (×20 objective).

Staining patterns of ERRs were graded on a scale of 0/+/++/+++ (+++ = increased staining compared with control, ++ = stained as control, + = decreased staining compared with control, 0 = unstained/negative) (×20 objective). CD3-positive T-cells were analyzed by counting the mean number of positive cells per field from 2–4 high-power fields (hpfs) (×40 objective). CD3-positive cells were graded as 1 = < 50 cells/hpf, 2 = 50–100 cells/hpf, 3 = > 100 cells/hpf. Scoring of each section was based on a consensus of two investigators and possible disagreements were resolved in a joint review.

4.2.6 Microbiome analysis (Study IV)

The swab samples were analyzed by using a Next-Generation Sequencing (NGS) method in order to identify bacterial RNA sequences. DNA extraction from the study samples was carry out with Macherey-Nagel NucleoSpin Tissue DNA extraction-kit (Macherey-Nagel Gmbh & Company KG, Germany), with extra bacterial lysis step. After DNA extraction, the DNA library was built by using "16S metagenomics sequencing preparation instructions; preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system". Thereafter, sequencing was performed with MiSeq (Illumina, USA).

Primers removal from amplicon sequencing data was done using cutadapt tool and amplicon sequence variants were inferred using DADA2 approach (Callahan et al., 2016). The statistical analyses were performed in R statistical software (version 3.5.3). Analyses were performed in three different settings:

1. All patients (n=30) versus controls (n=21). One case and two controls had been on antibiotics.
2. All patients (n=29) versus controls (n=19). Participants who had used antibiotics during the last month were excluded.
3. Patients with secondary vulvodynia (n=24) versus controls (n=19). Participants who had used antibiotics during the last month were excluded.

For more detailed description of preparing and analyzing the samples please see Study IV.

4.2.7 Statistical analysis (Studies I-IV)

In statistical analysis, version 23 of IBM SPSS statistics software was used in Studies I-II (IBM SPSS Statistics for Windows, Version 23.0. IBM Corp. 2015. Armonk, NY, USA) and version 24 of IBM SPSS statistics software in Studies III-IV (IBM SPSS Statistics for Windows, Version 24.0. IBM Corp. 2016. Armonk, NY, USA). A probability value of $p<0.05$ was considered statistically significant.

The Mann–Whitney U-test was used to compare patient-reported NRS values after different treatment modalities. Wilcoxon's signed-rank test was used to study the overall effect of combination of treatments on NRS values (Study I). The associations between number of outpatient clinic visits, patient's age, presence of co-morbidities, QoL and patient-reported NRS values was also analyzed by the Mann–Whitney U-test (Study I). Differences in patient-reported scores describing satisfaction with treatments given by different professionals (i.e. physicians, physiotherapists, trained nurses) were analyzed using Wilcoxon's signed-rank test (Study I). The Mann–Whitney U-test was used for statistical comparisons in Study II. Fisher's exact test and the Mann–Whitney U-test were used for statistical comparisons as appropriate (Study III).

Biostatistical analyses (Study IV) were performed with R statistical software (version 3.5.3). Statistically significances between LPV patients and controls were tested by means of a permutational multivariate analysis of variance (PERMANOVA) test using a Euclidean distance matrix implemented in the *adonis* function of R package *vegan*. The PERMANOVA test is based on the assumption that there is homogeneity of dispersion within compared groups. The validity of the assumption is then tested by analyzing multivariate homogeneity of group dispersions using the *betadisper* function in R package *vegan*. Differentially abundant taxa were determined using the DESeq2 package.

4.2.8 Ethics statement

The studies comply with the Declaration of Helsinki. Study protocols were reviewed and approved by Pirkanmaa Hospital District Ethics Committee. (Identification codes: R14037, R16053; R17081). Patients recruited to this study gave their written informed consent. The Finnish National Supervisory Authority for Welfare and Health gave its permission to use the vestibulectomy archives in Study III.

5 RESULTS

5.1 Retrospective patient cohort of vulvodynia patients 2003-2013 (Study I)

The response rate to postal questionnaires was 52.6%. Timespan between treatments the self-assessment was 1-11 years. Characteristics of the study population are shown in Table 10. The various treatments given are summarized in Table 11.

Table 10. Characteristics of the Study Population in study I (n=70)

Age, median (interquartile range)	30 (25-41)
Onset of symptoms (years), median (interquartile range)	20 (17.25-27.50)
Duration of symptoms before treatments (years), median (interquartile range)	1.0 (0.5-4.75)
Nulliparous, n (%), missing information n=2	54 (77.1)
Dyspareunia n (%) missing information n=1	64 (91.4)
Postmenopausal n (%)	12 (17.1)
Generalized pain, n (%)	14 (20)
Local pain, n (%)	56 (80)

Table 11. Different treatment modalities used for vulvodynia patients in study I

	n (%)
Desencitizing gel [1)]	58 (82.9)
Physiotherapy (biofeedback, TENS)	55 (78.6)
Sexual counseling by a trained nurse	52 (74.3)
Topical gabapentin 6%	38 (54.3)
Topical neuromodulation [2)]	23 (32.9)
Local injections to painful site [3)]	18 (25.7)
TCA [4)]	14 (20.0)
Surgery [5)]	13 (18.6)
Pregabalin 150-300 mg	10 (14.3)
Antibiotic or antifungal treatment [6)]	7 (10.0)
Laser treatment	3 (4.3)
Sacral neuromodulation	2 (2.9)

[1)] Lidocain gel to the painful area in vulva 30 minutes before intercourse.
[2)] Podophyllotoxin (5 mg/mL Wartec®) applied locally to tender points of vestibulum following 5% acetic acid application. Treated area was covered with a mild estrogen cream and covered with gauze pads until the next day.
[3)] 2-4 ml of cortisone (betamethasone) and long acting anesthetic agent (bupivacaine), both 50% and 50 %, injected submucously to the painful site.
[4)] Tri-cyclic antidepressant, amitriptyline 10-40 mg most commonly used
[5)] Modified posterior vestibulectomy, surgical removal of painful area
[6)] If diagnosed with yeast or bacterial infection
TENS = Transcutaneous nerve stimulation
TCA: Tri cyclic antidepressant

The most frequent treatment combinations were: desensitizing gel and physiotherapy (67.1 % received, n=47) and desensitizing gel, physiotherapy and sexual counselling (52,9% received, n=37). The median NRS value of vulvodynia-related pain was 8.0 (interquartile range [IQR] 8–9) before treatment and 4.0 after treatment (IQR 2–7). The effects of various treatments were associated with a significant reduction in NRS values before and after the treatments ($p < 0.001$). When the NRS scores after individual treatments between groups (treatment/no treatment) were compared, no differences were found.

A history of depression or bipolar disorder was not associated with poorer treatment outcome when comparing vulvodynia patients with and without psychiatric disorders (median reduction in NRS 2 vs. 4, $p = 0.274$; median pretreatment NRS 9, IQR 8–9 vs. 8, IQR 8–9 for patients with psychiatric vs. no psychiatric disorder, $p = 0.27$; and median post-treatment NRS 6.5, IQR 2.3–8 vs. 4, IQR 2–6.5 for patients with psychiatric vs. no psychiatric disorder, $p = 0.071$). Localized vs. generalized vulvodynia was not associated with treatment outcome (median reduction in NRS 4 [IQR 2–6] vs. 3 [IQR 1–7] for patients with local pain vs. generalized pain syndrome, $p = 0.763$).

When age was categorized using the median value as a cut-off point, the reduction in NRS score was lower after treatment in older patients (median reduction in NRS 2, IQR 1–6 vs. 5, IQR 2–7, p = 0.032). The median number of outpatient clinic visits was four (range 1–17, IQR 2–6). A greater number (≥ 6) of outpatient clinic visits was associated with a smaller reduction in NRS values (median reduction in NRS 2, IQR 1–5 vs. 4, IQR 2–7; p = 0.043). Age was not associated with the number of outpatient clinic visits (median number of visits four among patients of ≤ 30 and > 30 years of age, p = 0.179). The median time from onset of vulvodynia symptoms to initiation of therapy was one year (IQR 0.5–4.75), which was not associated with the treatment outcome (median reduction in NRS 4, IQR 2–7 vs. 3, IQR 1–7 for patients with < 1 year from onset of symptoms vs. ≥ 1 year, respectively, p = 0.352).

Patient satisfaction with different professionals was high: 77.1% of patients were satisfied with treatment given by physiotherapists, while the corresponding numbers were 51.5% for trained nurses (sexual counseling) and 65.7% for physicians. The median score for satisfaction with physiotherapists was 5 (IQR 4–5). The median satisfaction score for physicians was 4 (IQR 4–5) and for sexual counseling by trained nurses the median score was also 4 (IQR 3–5). The patients were more satisfied with treatments given by physiotherapists than physicians (p = 0.015). Satisfaction with physiotherapists was also higher when compared with trained nurses (p < 0.001). Satisfaction with physicians vs. trained nurses was not different (p = 0.172). Patients, who were satisfied with treatment given by physicians, reported higher reduction in pain scores (median reduction in NRS 4, IQR 2–7 vs. reduction in NRS 2, IQR 1–5.25), but the difference was not statistically significant (p = 0.053) in comparison with unsatisfied patients.

Most patients (67.1%) reported good QoL at survey. Self-reported pre-treatment pain scores (NRS values) were not associated with the QoL (median NRS 9, IQR 8–9 vs. 8, IQR 8–9 for patients reporting good QoL vs. poor QoL; p = 0.327). Patients reporting good QoL reported lower NRS scores after treatment (median reduction in NRS 6, IQR 3–7 vs. 1, IQR 0–2, p < 0.001; and median NRS scores after treatment 3, IQR 2–5 vs. 7, IQR 6–8, p < 0.001 for patients reporting good QoL vs. poor QoL, respectively.

5.2 Surgically vs non-surgically treated LPV patients (Study II)

From the original study population (n = 66), 36 patients returned the postal questionnaire during the study period (55%). The response rate to postal

questionnaires was different between surgical and non-surgical groups; in the surgical group it was 81.3% and in the non-surgical group 46.0% ($p = 0.020$). The median follow-up time at data-analysis point 2 was 36 months (IQR 24–36). The patients' demographic data and various treatments received are shown in Table 12. At data-analysis point 1 (two months after commencing the treatments), the patients in surgical groups were older than in non-surgical group (Table 10). The various treatments given to study patients are summarized in Table 10. The most frequent (received by > 50% of the patients) combination of non-surgical treatments consisted of local treatment (lidocaine and/or gabapentin), physiotherapy, and sexual counseling in both patient cohorts. At data-analysis point 2 the two treatment groups differed with respect to the frequency of sexual counseling (Table 12). At data-analysis point one the two groups did not differ regarding the received treatment modality.

Table 12. Demographic data and treatments received by LPV patients

Data analysis point 1: Review of medical records

	All LPV patients	Non-surgically treated patients	Surgically treated patients	p-value [1]
Number of patients	66	50	16	N/A
Age, median (Inter-quartile range, IQR)	28 (25-33)	27 (24-32.3)	30.5 (26.5-38.3)	0.048
Nulliparous % (n)	95.5 (63)	94 (47)	100 (16)	0.320
Premenopausal % (n)	98.5 (65)	100 (50)	93.8 (15)	0.077
Local treatments % (n) [2]	100 (66)	100 (50)	100 (16)	1.000
Tri-cyclic antidepressant (TCA) or anticonvulsant [3]	15.2 (10)	12.0 (6)	25.0 (4)	0.210
Physiotherapy (including TENS)	90.9 (60)	92.0 (46)	87.5 (14)	0.589
Sexual counseling by a trained nurse	75.8 (50)	80.0 (40)	62.5 (10)	0.158
Topical treatments [4]	22.7 (15)	18.0 (9)	37.5 (6)	0.108
Local injections to the painful site [5]	16.7 (11)	16.0 (8)	18.8 (3)	0.799

Data analysis point 2: Review of medical records and postal questionnaire

	All LPV patients	Non-surgically treated patients	Surgically treated patients	p-value [1]
Number of patients	36	23	13	N/A
Age, median (Inter-quartile range, IQR)	28.5 (25-32)	27 (24-29)	29 (26.5-33)	0.062
Nulliparous % (n)	86 (31)	82.6 (19)	92.3 (12)	0.480
Premenopausal % (n)	100 (36)	100 (23)	100 (13)	1.000
Local treatments % (n) [2]	100 (36)	100 (23)	100.0 (13)	1.000
Tri-cyclic antidepressant (TCA) or anticonvulsant [3]	16.7 (6)	13.0 (3)	23.1 (3)	0.350
Physiotherapy (including TENS)	88.9 (32)	91.3 (21)	84.6 (11)	0.460
Sexual counseling by a trained nurse	77.8 (28)	87.0 (20)	61.5 (8)	0.038
Topical treatments [4]	19.4 (7)	8.7 (2)	38.5 (5)	0.050
Local injections to the painful site [5]	11.1 (4)	8.7 (2)	15.4 (2)	0,769

[1] P-value between surgical and non-surgical group

[2] Lidocaine gel to the painful area in vulva 30 minutes before intercourse or gabapentin 6% cream applied twice a day to the painful area for 6-8 weeks

[3] Amitriptyline 10-40 mg most commonly used TCA or pregabalin 150-300 mg

[4] Podophyllotoxin (5 mg/mL) applied locally to tender points of vestibulum following 5% acetic acid application. Treated area was covered with a mild estrogen cream and covered with gauze pads until the next day.

[5] 2-4 ml of betamethasone and long acting anesthetic agent (bupivacaine), both 50% and 50 %, injected submucously to the painful site.

TCA = Tri-cyclic antidepressant

TENS = Transcutaneous nerve stimulation

At data-analysis point 1, median pretreatment NRS scores were similar between the surgical and non-surgical (i.e. combination of treatments without surgery) groups (Table 13). Median post-treatment NRS scores assessed by a physician in the two treatment groups were different (Table 13, Data analysis point 1). At data analysis point 2, the physician-assessed NRS score before treatment differed, surgically treated group reported more intense pain than non-surgical group (Table 13). Similarly, post-treatment NRS scores assessed by a physician were different at data analysis point 2. Self-reported NRS scores before and after treatments did not differ between the groups (Table 13) at data analysis point 2. Among the LPV patients who did not respond to postal questionnaires (n = 30) the median pretreatment NRS

score collected from the patient records was 9 (IQR 8–9.5, missing data n = 13), and the median two-month post-treatment NRS score was 5 (IQR 2.25–8, missing data n = 14). Non-responders vs. responders to the postal questionnaires did not differ in respect of the pre- and post-treatment NRS scores derived from medical records (p = 0.291, p = 0.592 respectively).

Table 13. Assessment of pain in Study II patients

Data analysis point 1: Review of medical records	**All LPV patients**	**Surgical treatment**	**Non-surgical treatment**	**p-value [1)]**
Number of patients	66	16	50	-
NRS before treatments, assessed during the cotton-swab test	9 (7.25-9), no data (n.d.) n=22	9 (8-9.5), n.d. n=4	9 (7-9), n.d. n=18	0.114
NRS after treatments, assessed during the cotton-swab test	5 (2-8), n.d. n=24	2 (2-4), n.d. n=5	7 (4-8), n.d. n=19	0.008
Data analysis point 2: Review of medical records and postal questionnaire	**All LPV patients**	**Surgical treatment**	**Non-surgical treatment**	**p-value [1)]**
Number of patients	36	13	23	-
NRS before treatments, assessed during the cotton-swab test	9 (7-9), n.d.=9	(8-10), n.d.=2	8 (7-9), n.d. n=7 9	0.014
NRS after treatments, assessed during he cotton-swab test	5 (2-7), n.d. n=10	2 (2-4), n.d. =3	7 (4.5-8), n.d. =7	0.005
Self-reported NRS before treatments in the postal questionnaire	8 (8-9)	8 (8-9)	8 (7-9)	0.661
Self-reported NRS after follow-up in the postal questionnaire	3 (2-5.75)	2 (2-5)	4 (3-6)	0.184

[1)] p-value between surgical and non-surgical group NRS=Numerical rating scale n.d.=No data

QoL assessed by the RAND-36 questionnaire at the follow-up after treatments at the analysis point 2 did not differ between the surgical and non-surgical groups in any of the eight multi-item dimensions (Table 14, Figure 8). However, non-surgically treated LPV patients had lower QoL in general health (62.1, SD 23.9 vs. 74.9, SD 17.8, p = 0.018), emotional role functioning (56.5, SD 43.9 vs. 76.7, SD 34.0, p = 0.049) and pain (64.7, SD 24.5 vs. 80.5, SD 21.2, p = 0.005) when compared with healthy women aged 25–34 from the Finnish national register, while surgically treated patients did not differ from the general age-matched population (Table 14, Figure 8).

Table 14. Quality of life after follow-up in different RAND-36 dimensions in Study II

	Surgical treatment	Non-surgical treatment	p-value [1)]
Physical functioning / health, mean (SD)	95.4 (15.20)	92.4 (14.45)	0.243
Physical role functioning, mean (SD)	84.6 (33.13)	69.6 (43.92)	0.278
Emotional role functioning, mean (SD)	66.7 (40.82)	56.5 (46.53)	0.498
Vitality, mean (SD)	58.1 (16.65)	51.5 (23.95)	0.518
General mental health, mean (SD)	68.9 (22.87)	65.7 (21.77)	0.416
Social functioning, mean (SD)	79.8 (19.46)	72.3 (27.94)	0.485
Pain (SD)	75.2 (26.76)	64.7 (24.50)	0.144
General health perceptions, mean (SD)	63.9 (21.03)	62.2 (23.88)	0.974

[1)] P-value between surgical and non-surgical treatment groups

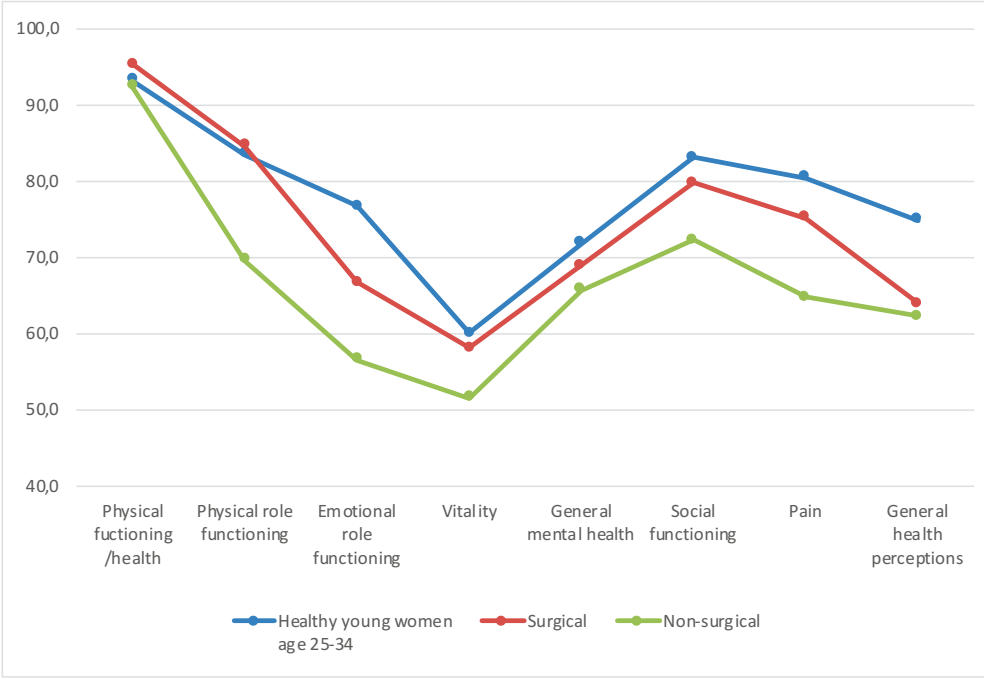

Figure 8. Quality of life assessed by RAND-36.

The complication rate after surgery was 18.8% (three patients out of 16 surgically treated patients). One patient was readmitted seven days after surgery, because of partial wound dehiscence. The wound healed completely at the two-month follow-up visit. Another patient had heavy postoperative pain and was readmitted to hospital on the third postoperative day. Two months after surgery the patient was still suffering from pain, while after one year of follow-up the pain in the vulvar area

was reported to have "transformed into a neuropathic pain" and the patient was treated with oral gabapentin, which resulted in sufficient pain relief. The third patient suffered from severe pain immediately after surgery and had to stay overnight at the hospital. However, at two-month follow-up the pain score was assessed as "0" by the operating physician.

5.3 Estrogen-related receptors and other biomarkers in LPV patients (Study III)

The demographic data of study patients is shown in Table 15. LPV patients were more often nulliparous and younger than control patients (Table 15).

Table 15. Patient characteristics in Study III

	LPV patients (n=12)	Healthy controls (n=15)	P-value
Age, median (IQR)	27 (23.25-34.75)	39 (34-44)	0.016
Premenopausal, n(%)	11 (91.7)	15 (100)	0.444
Nullipara, n (%)	10 (83.3)	6 (40.0)	0.047
Combined contraceptives	1 (8.3%)	2 (13.3)	1.000
Progestin only	2 (16.7)	1 (6.7)	0.569
Symptom duration in months, median (IQR)	20.5 (12-23.5)	N/A	N/A

No specific pathological diagnostic abnormality was diagnosed in LPV patients or in controls in hematoxylin staining. In three LPV samples chronic non-specific inflammation was detected. Normal vestibulum samples from the controls stained all ERR isoforms analyzed in this study uniformly. In both the LPV and control samples, ERRα and ERRß expression was both nuclear and cytoplasmic, while ERRγ showed only nuclear staining in IHC. Overall ERRß staining (both nuclear and cytoplasmic) was more distinct in LPV samples than in controls (Table 16, Figure 9). No differences were found in the levels of ERRα and ERRγ expression (Table 16). Staining of ER, PR and CD3 was similar in LPV patients and controls (Table 16).

Table 16. Stainings of ERα, PRα, CD3, ERRα, ERRß and ERRγ in Study III

	LPV patients (n=12) [1]	Healthy controls (n=15) [2]	p-value
ER			
Not stained	0	0	
Less than 10%	0	0	0.181
11-50%	0	2	
51-100%	12		
PR			
Not stained	0	0	
Less than 10%	1	5	0.078
11-50%	3	4	
51-100%	8	5	
CD3			
<50 cells / HPF	0	0	
50-100 cells / HPF	1	4	0.236
>100 cells / HPF	10	10	
ERR alpha			
Stained less than controls	0	0	
Stained as controls	11	14	1.000
Stained more than controls	0	0	
ERR beta			
Stained less than controls	0		
Stained as controls	6	14	0.006
Stained more than controls	5		
ERR gamma			
Stained less than controls	0	0	
Stained as controls	11	14	1.000
Stained more than controls	0	0	

[1] One vestibulectomy sample was sufficient only for ER ja PR stainings.

[2] One of the control samples taken did not contain epithelium, sample was excluded from the analysis

HPF = high power field

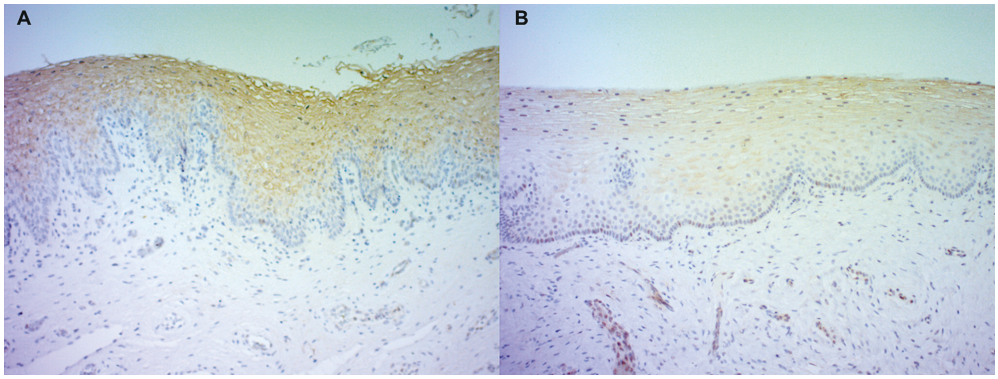

Figure 9. Examples of ERRß staining in A) LPV patient's sample, B) control

5.4 Microbiome in LPV patients vs controls (Study IV)

The demographic data of all LPV patients, secondary-LPV patients and controls are shown in Table 14. All LPV patients had slightly lower BMI, they were younger and more frequently nulliparous than the controls (Table 17). The same hold true when compared secondary-vulvodynia patients to controls. At the time of the sampling, ten out of all 30 LPV patients had an undefined day of the menstrual cycle (no bleeding/irregular bleeding) for the following reasons: one was postmenopausal, one was using a combined oral contraceptive and the rest were using either progestin-only pills or a levonorgestrel-releasing intrauterine device (LNG-IUD). Seven out of the 22 secondary-LPV patients had an undefined day of the menstrual cycle (no bleeding/irregular bleeding) due to the following reasons: one was postmenopausal, one was using a combined oral contraceptive and the rest were using either progestin-only pills or an LNG-IUD. Three of the 21 controls had undefined bleeding patterns; two did not have periods as a result of cervical stricture or Ashermann's syndrome. One control subject was amenorrhea as a result of use of progestin-only pills. Swab samples were taken on average (median) on day 11 of the menstrual cycle (n = 38, IQR 8.75–19.75) among all the women. Most of the LPV patients had their samples taken on day 15 or before (65.8%). Most of the secondary-LPV patients also had their samples taken on day 15 or before (53.5%).

Table 17. Demographic data of all LPV patients, secondary LPV patients and controls (Study IV)

	All LPV patients (n=30)	Secondary LPV patients (n=22)	Control (n=21)	p-value [1]	p-value [2)]
Age, median (IQR)	24.5 (20.0-32.5)	26.5 (20.0-34.0)	37 (32-41.5)	0.000	0.001
Premenopausal, n (%)	29 (96.7)	21 (95.5)	21 (100)	0.403	0.329
Nulliparous, n (%)	28 (93.3)	20 (90.9)	8 (38.1)	<0.001	0.000
BMI, median (IQR)	22.3 (20.28-24.78)	24.27(20.9-27.9)	24.8 (23.6-32.2)	0.002	0.021
Non-smokers, n (%)	28 (93.3)	20 (90.9)	19 (90.5)	0.712	0.961
Primary vulvodynia, n (%)	8 (26.7)	N/A	N/A	N/A	N/A
Secondary vulvodynia, n (%)	22 (73.3)	N/A	N/A	N/A	N/A
Previous use of antibiotics (last month)	1 (3.3)	1 (4.5)	2 (9.5)	0.336	0.527
Day of the menstrual cycle, median (IQR)	14.5 (10-23.75)	14 (10.0-23.0)	10 (7.5-13.25)	0,051	0.106
The use of hormonal contraception [3)], n (%)	18 (60)	12(54.5)	8 (38.1)	0.16	0.285

N/A = not applicable

[1)] Between all LPV patients and controls

[2)] Between secondary LPV patients and controls

[3)] Including combined oral contraceptives, patches, ring and progestin only pill and levonorgestrel containing intrauterine device

Comparison of microbial communities in patients and controls is presented in Table 18. Differences were found only when comparing secondary LPV patients and controls (Table 18).

Table 18. Comparison of overall microbial communities between LPV patients and controls (p-values)

Analysis	All LPV patients and controls (Setting 1)	All LPV patients and controls [1] (Setting 2)	Secondary LPV patients and controls [1] (Setting 3)
Multivariate homogeneity of group dispersions analysis (p-value)	0.30	0.31	0.05
Permutational multivariate analysis of variance (p-value)	0.08	0.13	0.04

[1] Participants with previous antibiotic use (1 month) were excluded from the analysis

When comparing alpha diversity, which refers to the average diversity in a specific area (e.g. vulvar vestibulum), there was no difference between cases and controls (Chao 1: p-value 0.14, Shannon: p-value 0.06, Simpson: p-value 0.08).

Beta diversity, referring to the ratio between alpha diversity and regional diversity, was different in secondary-LPV patients vs. controls (Permanova, R2: 0.04, p-value 0.05).

The final differential abundance analysis at taxon level showed that statistically significant differences were found when the secondary LPV patients were compared to the controls at 31 taxa level (Table 15).

Table 15. Results from differential abundance analysis between secondary LPV patients and controls

More abundant in controls:
Aerococcus Christensenii (p=0.018793)
Anaerococcus Murdochii (p=0.000/ 2,34 x 10^{-5})
Dialister sp (p=0.030585)
Ezakiella sp (p=0.000/ 5,44 x 10^{-8})
Fastidiosipila sp (p=0.000/ 2,2 x 10^{-9})
Gardnerella sp (p=0.001993)
Gemelia Asaccharolytica (p=0.000/ 2.34 x 10^{-5})
Lactobacillus Crispatus (p=0.007259)
Lactobacillus sp I (p=0.000/ 1.2x 10^{-14})
Lactobacillus sp II (p=0.000/ 1.4 x 10^{-10})
Megasphaera sp (p=0.000/ 1.63x 10^{-7})
Megasphaera sp (p=0.006146)
Mobiluncus curtisii (p=0.001562)
Parvimonas sp (0.000/ 3.75 x 10^{-5})
Peptoniphilus Duerdenii (p=0.00082)
Peptoniphilus Massiliensis (p=0.000/ 9.31 x 10^{-5})
Porphyromonas (p=0.000772)
Porphyromonas Uenonis (p=0.000/ 5.46 x 10^{-5})
Prevotella Bergensis (p=0.01317)
Prevotella Bivia (p=0.000/ 1.71 x 10^{-9})
Prevotella Disiens (p=0.000/ 2,14x 10^{-6})
Prevotella sp (p=0.000/ 2,34x 10^{-5})
Prevotella sp II (p=0.000107)
Slackia Exigua (p= 0.000/ 6.24 x 10^{-5})
Sneathia sp (p=0.000/ 0.005401)

More abundant in the secondary LPV samples:
Fastidiosipila sp (0.013257)
Gardnerella vaginalis (p=0.000/ 4.65x10^{-5})
Lactobacillus sp I (p=0.000/ 6.35 x 10^{-6})
Peptoniphilus sp (p=0.0009151)
Prevotella Amnii (p=0.000/ 7.29x 10^{-11})
Sreptococcus sp (p=0.000123)

Bacteria taxa that were increased among patients were Gardneralla vaginalis ($p<0.0001$), Peptoniphilus sp ($p<0.0001$), Prevotella amnii ($p<0.0001$), Streptococcus sp ($p=0.0001$), Lactobacillus sp I ($p<0.0001$) and Fastidiosipila ($p=0.013257$). Several bacterial species were more common abundant among healthy controls reflecting reduced bacterial diversity among patients.

6 DISCUSSION

6.1 Etiology (Study III, Study IV)

6.1.1 Steroid receptors and CD3 positive T-cells

According to this study, staining patterns of ER and PR and the number of T-cells were similar in LPV patient samples and controls. Previously, only a few studies have concerned the expression of steroid receptors in LPV, with controversial results. An upregulation of the expression of ER in the vestibula of LPV patients has been reported (ERα) (Johannesson et al., 2008; Leclair, et al. 2011)- or no difference has been found (Goetsch et al., 2010; Leclair et al., 2013). The different results obtained, however, may be partly explained by differences in study setting (primary/secondary LPV, postmenopausal/premenopausal LPV) and categorization. In the present study, the standardized IHC methodology validated in breast-cancer diagnostics (Hammond et al., 2010) was used. The present results regarding PR expression and CD3-positive T-cells were similar to those in previous studies showing no difference between LPV and controls (Johannesson et al., 2008; Tommola et al., 2015). Even if hormonal or inflammatory factors may have a role in the pathogenesis in LPV, they cannot be directly shown at protein level by IHC methodology based on the findings in the present thesis.

6.1.2 Expression of ERR's

In Study III different ERR isoforms expressions were found in vestibula between LPV and controls. This was a novel finding. As for expression of ERR isoforms, the expression of ERRß was more pronounced in the vestibula of LPV patients compared to controls. However, no differences in ERRα and ERRγ expressions were found. The expression of different ERR isoforms have been studied in various gynecological diseases. Levels of ERRα decrease in the pathogenesis of vulvar cancer in an LSA-positive background (Lagerstedt et al., 2015). In contrast, in another study,

ERRα mRNA was upregulated in ovarian cancer, while levels of ERRβ and ERRγ were undetectably low (Fujimoto et al., 2007). Expression levels of ERRα and ERRγ have been shown to decrease in endometriotic lesions, but this was not reported in the case of ERRβ (Cavallini et al., 2011). In endometrial cancer, increased expression of ERRα showed to be associated with advanced clinical stage and histologically more aggressive disease. Furthermore, ERRα silencing has resulted in reduced cell proliferation in vitro (Matsushima et al., 2016). All these findings suggest that dysregulation of different ERR isoforms may be active in various gynecological diseases.

A decline in ERRα and ERRγ expression has been reported in the vaginal epithelium of postmenopausal women, but this was not observed for ERRβ (Cavallini et al., 2008). In a study where ERRβ expression was studied in normal human endometrium, ERRβ mRNA and protein were found to be expressed in healthy human endometrium, although ERRβ protein was mainly localized in the nuclei of both stromal and endometrial cells (Bombail et al., 2008). In the present thesis, in contrast, ERRβ was found to be expressed uniformly both in the cytoplasm and nuclei in healthy vulvar epithelium. This may suggest a difference in the function of ERRβ between normal endometrium and vulva but needs to be further investigated.

Estrogenic activation has been shown to modulate ion-channel activation, either stimulating or inhibiting the channels (Kow et al., 2016). Inflammation is one possible trigger in peripheral sensitization (von Hehn et al., 2012), and inflammatory mediators such as cytokines can cause a reduction in threshold and an increase in excitability of peripheral nociceptors (Kow et al., 2016; von Hehn et al., 2012). Hence, changes in hormonal activity can theoretically induce reduction in thresholds for nerve activation through changes in potassium and sodium channels. This may lead to the peripheral sensitization in LPV, parallel to the mechanism of how nerve injury can lead to peripheral sensitization (von Hehn et al., 2012). ERRγ has also been found to regulate transcriptional activation of potassium channel genes during human trophoblast differentiation in vitro (Luo et al., 2013) and thus it is possible that other ERRs may be involved in the pathogenesis of chronic pain conditions such as LPV by modulating the potassium and sodium channels.

The hormonal and inflammatory factors mentioned above may be interrelated in the pathogenesis of LPV, since estrogen has also been shown to modulate immune responses by restricting neutrophil accumulation to the site of inflammation, attenuating the release of pro-inflammatory mediators, and regulating estrogen receptor gene expression in T-, B- and dendritic immune cells (Nadkarni et al., 2013).

6.1.3 Differences in the vulvar microbiome

There are relatively few studies on microbiome of vulvar vestibulum, however, the vestibular microbiome resembles that of the vagina, suggesting that vaginal excretions are an important source of the vestibular microbiome (Jayaram et al., 2014). The bacterial microbiomes of the labia majora and labia minora also resemble that of the vagina (Shiraishi et al., 2011), and thus it is probable that secretions from the vagina leak and coat the vestibular surface, resulting in the same dominant bacterial genera both in the vagina and vestibulum. This discussion is therefore based mainly on studies of the vaginal microbiome.

In the present thesis there were differences in a total of 31 various bacteria species in the vulvar vestibulum between LPV patients and controls (Table 15). The finding was significant only when primary-LPV patients were excluded from the comparison, that is between secondary LPV patients and controls, supporting the theory that secondary vulvodynia might stem from different pathology (Goetsch, 2010; Leclair, 2011; Pukall, 2016) primary vulvodynia. Changes in the bacterial microenvironment of the vulva, caused, for example, by inflammation, might contribute to the pathogenesis of secondary LPV by mechanisms described above (Section 6.1.2).

In one previous study, *Lactobacillus crispatus* was not found in samples from vulvodynia patients when assessed by means of quantitative PCR (Ventolini et al., 2013). This finding resembles the results of the present study where *Lactobacillus crispatus* was found more often in the samples of the control women compared to LPV patients. Jayaram et al. (2014) also found that *Lactobacillus. crispatus* seemed to be dominant in controls than patients, even though the difference was not of statistical significant (Jayaram et al., 2014). *Lactobacillus gasseri* has been present only in samples from vulvodynia patients (Jayaram et al., 2014) and *Lactobacillus iners* has been dominant at a higher frequency in patients than in controls (Jayaram et al., 2014). Group B betahemolytic streptococcus has been associated with vulvar pain, superficial fissures and minimal erythema of the vulvar skin (Mirowski et al., 2012), typical features also for LPV. In the present study, particular streptococcus species were more abundant in vestibular samples of LPV patients. Unfortunately, the precise bacterial species could not be detected in confirmatory assays. Whether or not these preliminary findings contribute to the pathogenesis of LPV remains thus to be determined in future studies.

The gut microbiome is known to regulate estrogen levels, and estrogen has also been shown to have an impact on the gut microbiome (Baker et al., 2017). Particular

bacterial species can colonize both the reproductive tract and gastrointestinal tract, suggesting that the rectum could be the origin of bacterial species that commonly colonize the vagina (Freitas et al., 2017). Estrogen has been shown to modulate the vaginal microbiome by increasing the number of lactobacilli and lowering the pH, which contribute positively the health of vagina (Muhleisen et al., 2016). However, the total lactobacilli load without considering the specific bacterial species is not a good parameter to assess the health status of the vagina (Biagi et al., 2009; Jespers et al., 2012). Different patterns of inflammation are known to be activated within each CST according to the dominant lactobacillus species, and non-lactobacillus bacteria can contribute to the pathogenic mechanism behind inflammation (De Seta et al., 2019). With known theories of hormonal signaling contributing to the pathogenesis of LPV, estrogen, once again, could be a possible link in the inflammatory and hormonal interplay in the pathogenesis of LPV (Chapter 6.1.2).

The presence of aerobic vaginitis has been found to correlate positively with the severity of LPV (Donders et al., 2018). It is an asymptomatic condition in 10–20% of women. It is dominated by aerobic microbiota (e.g. *Escherichia. coli*, group B streptococci, *Stafylococcus aureus*) and decreased numbers of lactobacilli (Jackie et al., 2018). Atrophic vaginitis in lactating women is probably a variant of aerobic vaginitis, and more severe forms of aerobic vaginitis and desquamative inflammatory vaginitis (DIV) represent probably the same condition (Jackie et al., 2018). At the moment, with known alterations in the vaginal microbial constitution, clinical practice could include wet-mount microscopy and, for example, diagnosed aerobic vaginitis could be treated properly. In the future, probably in ten years, it may be possible to use methods such as bacterial DNA sequencing/ NGS to assess vaginal and vulvar microbiome as a part of clinical practice.

The LPV patients in the present study sample were younger than the controls and variations in the microbiome occur depending on age (Costello et al., 2009), which may have had an effect on the results. However, all patients but one were clearly premenopausal, and such a bias is inevitable in this kind of clinical study setting. In addition, the BMI of the patients and controls varied, the patients had lower BMI. A BMI higher than 30 kg/m^2 has been shown to be associated with *Finegoldia-* and *Corynebacterium*-dominant vulvar microbiota, and *Lactobacillus* species represent the dominating flora in women with BMI lower than 30 kg/m^2 (Vongsa et al., 2019). However, in the present study population both study groups had median BMI values of < 25 kg/m^2. The day of the menstrual cycle and the use of hormonal contraceptives can also affect the microbial flora (Gupta et al., 2019). However, there was no difference between groups in these variables. Patients with recent antibiotic

medication (< 1 month previously) were excluded from the final analysis to prevent bias linked to the use of antibiotics.

6.2 Therapeutic options (Study I, Study II)

6.2.1 Treatment outcomes in the University-Hospital cohort (Study I)

Combined therapies given by a multidisciplinary team were efficient in reducing LPV-related pain by half (Study I). However, none of the individual treatment was associated with better outcome. In the literature multidisciplinary treatments that combine physiological and psychosexual treatments are recommended (Backman et al., 2008; Brotto et al., 2015; Goldstein et al., 2016; Nunns et al., 2010), although no standardized model of multimodal treatment exists (Backman et al., 2008). Therefore, no consensus of opinion as to which part of the multimodal treatment is the most beneficial to which patient has been verified (Goldstein et al., 2016). Different study centers combine different treatments with varying numbers of appointments, including group sessions in some studies (Brotto et al., 2015). In those studies in which peer-group sessions are also included as part of the treatment protocol, it is difficult to judge whether the therapeutic effect can partly be explained by the nonspecific therapeutic effect of the group rather than the treatments themselves (Brotto et al., 2015). In the present study group session were not included and for every patient the number of appointments with various professionals was individualized according to the patients' needs and motivation.

The most frequent combination of treatments in this study was desensitizing gel and physiotherapy: it was given to 67.1 % of the study patients (Figure 10). Further, this treatment combination added with sexual counseling was the second most frequent combination (52.9% received) (Figure 10). The results of the present study suggests that most of the treatment benefit is received with these combinations. Retrospective study cohort, and lack of 'pure' control group unfortunately prevents any further conclusions of the most efficient parts of multimodal treatment.

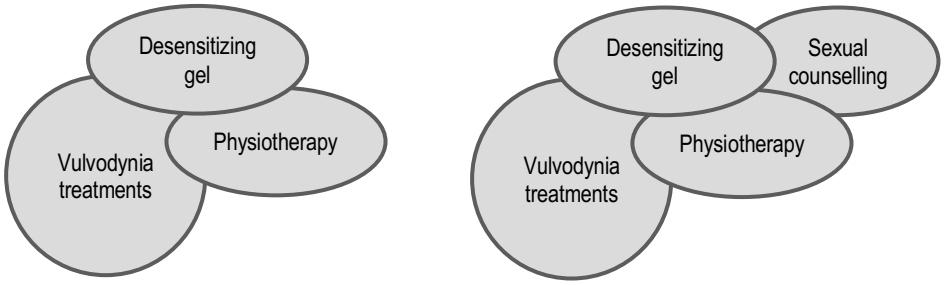

Figure 10. The most frequent treatment combinations in Study I

6.2.2 Treatment outcomes after combined treatments with and without surgery (Study II)

In Study II, where LPV patients' outcomes (pain) were reported after a median of 36 months of follow-up, the self-reported pain (NRS measures) did not differ after the follow-up. Previously, it has been reported that median pain measured by VAS decreased from eight to two in surgically treated patients (Tommola et al., 2011), which is in line with the present results concerning surgical treatment of LPV (self-reported median NRS score eight [IQR 8–9] before treatment and median NRS score two [IQR 2–5] after treatment). The results in regard to the pain measured by NRS straight after treatment during a cotton-swab test (median NRS score in conservative group seven [IQR 4.5–8] vs. surgery group median two [IQR 2-4]) were in favor of surgical treatment. However, this kind of study setting, where the patients visit the physician (operating surgeon) after the procedure and the physician reports the pain (NRS measures) are certainly not free of bias. Retrospectively reported NRS scores by the patients, however, did not differ after treatments.

There is little evidence that addition of multimodal treatments to surgery is, in fact, more efficacious than surgery alone (Schover et al., 1992; Weijmar Schultz et al., 1996). Schover et al. (1992) studied 45 women with LPV of whom 38 had vestibular surgery for LPV and they found that the patients who participated in psychological evaluation or postoperative sexual therapy had better outcomes than the patients who did not (Schover et al., 1992). All patients who did not improve had refused postoperative counseling. The researchers concluded that patients refusing psychological intervention may be poor candidates for surgery (Schover et al., 1992).

Vestibulectomy in treating refractory LPV cases is probably the best-documented treatment of LPV in the literature and well in favor of the procedure (Goldstein et

al., 2016; Tommola et al., 2010, 2011), with success rates of 60–90% (Landry et al., 2008). The limited number of randomized controlled studies (Bergeron et al., 2016; Weijmar Schultz et al., 1996) raises the question of a possible placebo effect of vestibulectomy, as reported in relation to various medical treatments of LPV (Miranda Varella Pereira et al., 2018). Sham surgery has been found to be as efficient as actual surgery, for example, in knee surgery (arthroscopic partial meniscectomy) (Sihvonen et al., 2013). However, Bornstein et al. (Bornstein et al., 1995) compared the effects of vestibuloplasty (denervating the mucosa in the vestibulum, n = 10) without removing any tissue with perineoplasty (removal of vulvar vestibulum, n = 11) and they found that vestibuloplasty without removal of the painful tissue was not sufficient as regards pain relieve of LPV patients, while perineoplasty cured almost all patients (nine out of ten). On the basis of this small study sample (Bornstein et al., 1995) it seems that removing the painful tissue is one of the key factors in successful outcome of various types of vestibulectomy.

The present results concerning conservative treatment with and without surgery in long-term follow-up were similar to those in a previous study (Tommola et al., 2012) in which surgery was found to be a valid option for those who do not respond to conservative treatments. The investigators (Tommola et al., 2012) found that long-term sexual well-being was good and VAS scores for pain decreased similarly in both surgical and non-surgical groups. However, long-term follow-up studies such as the present one cannot rule out the possibility of spontaneous remission of vulvodynia, as in one follow-up study, only 10% of patients suffered from constant pain during a two-year period of follow-up in a population-based observational study (Reed et al., 2016).

6.2.3 Number of outpatient visits as a predictor of treatment outcome (Study I)

In the present study, the median number of outpatient visits related to vulvodynia was four. In a recent database study from U.S. (Lua et al., 2017), a mean outpatient visits during a follow-up of one year after the initial diagnosis of vulvodynia was 20.9. This difference may reflect the different health-care systems in Finland and in the U.S., that is public health care system vs. insurance-based system. Yet another study (Xie et al., 2012), which was based on self-reporting of the visits, showed that vulvodynia patients visited outpatient clinic for a mean of 4.4 times over a six-month-period, similar to the findings in the present study although under a shorter

time period. As for the cumulative number of visit, in the present study patients with≥6 office visits had worse treatment outcome than patients with <6 visits. This result, clinically, is easy to understand. Patients with insufficient symptom relief repeat the visits plausible with a hope of a more efficient treatment. However, considering cost-efficiency and the limited resources, it might not be beneficial to increase the number of visits in a tertiary center without a prompt strategy to manage these patients. The highest number of office visits in our study sample was 17.

Consequently, the higher number of visits did not seem to add any value in respect of decreasing the pain. Should there be a prompt decision after for example 6 visits to direct the patient with GUV to a pain unit in University hospital setting or triage the patient with LPV to surgery would be of interest. Different types of vulvodynia treatment algorithms have been proposed (De Andres et al., 2016; Stockdale et al., 2014) including surgery but no recommendations over treatment duration is given. In the present study, the relatively high number of office visits in some cases might reflect the time where the original study sample from Study I was collected (2003-2013). The knowledge of vulvodynia and establishment of the vulva clinic (2009) has made the treatment more standardized and referral of patients to specialized physicians who are familiar with the condition has probably reduced the number of visits.

6.3 Quality of life (Study II)

The QoL of LPV patients treated by means of combined therapies with and without surgery did not differ in any of the eight QoL dimensions of the RAND-36 questionnaire. When conservatively treated LPV patients were compared with women of the same age in the general population, the patients seemed to suffer from poorer QoL in general health, emotional-role functioning and pain dimensions. The QoL of surgically treated patients did not differ from general population in any dimensions.

The effect of vestibulectomy on QoL was described in a retrospective study by Bohm-Starke et al. (2008), where QoL was improved from a median VAS score of 0.5 to 6.5 in patients treated with vestibulectomy, after a median follow-up period of 41 months (Bohm-Starke et al., 2008). Tommola et al. (2012) reported that conservatively and surgically treated LPV patients reached the same level of sexual and partnership satisfaction during long-term follow-up (47 months in the surgical group, 77 months in the conservative treatment group) in their observational case-

control study. This finding was similar to the present results, showing no difference in QoL between the two treatment groups. However, a difference in QoL was detected in three different dimensions between the conservatively treated LPV group and healthy women, while the surgically treated LPV patients reported similar QoL as the age-matched population. Previously it has been shown that vulvodynia has a detrimental effect for QoL, more than some other chronical conditions. For instance, in a study by Xie et al. the patients with vulvodynia had lower QoL than kidney-transplant recipients or patients with prior osteoporosis-related fractures (n = 174, QoL measured by means of Euro QoL-5 dimensions) (Xie et al., 2012). Vulvodynia, being a chronic pain condition, is known to affect a patient's life negatively (Arnold et al., 2006), but QoL even after various treatments seems to remain inferior compared with that in healthy age-matched women. This indicates that there is still an urgent need to develop better treatment modalities in order to improve these patients' QoL.

The findings reported in this thesis imply that conservative treatment followed by surgery may be superior to conservative treatment alone with respect to pain management, but the observed difference in pain management do not translate into a difference in QoL between the surgical and non-surgical treatment groups. The finding of inferior QoL in conservatively treated patients compared with women in the general population, however, needs to be confirmed in a larger study setting before any final conclusions can be drawn.

6.4 Strengths and limitations

In Study I, concerning a retrospective patient cohort, 70 patients returned the postal questionnaires and were included in the final analyses (52.6% of the original patient cohort). The percentage of responding patients is somewhat low, which could result in selection bias in the results. Being a single-center study in Finland's second biggest University Hospital, the study sample can be considered to be representative, although compared with studies conducted in bigger hospitals (for example in the U.S), the number of study participants is relatively low, which may have led to type I error in the statistical analysis. Another possible weakness of Study I is the lack of drop-out analysis. It is reasonable to assume that this was a source of selection bias in the final study population. After a relatively long timespan, retrospectively asked pain assessment before and after treatments is not as reliable as in a prospective study setting.

Study II concerned 16 surgically treated patients (13 of whom responded to the questionnaire) and 50 conservatively treated patients (23 of whom responded to the questionnaire). The sample size in Study II is admittedly small and this may definitely cause bias when interpreting the results. However, this reflects the rarity of surgical treatment of local provoked vulvodynia, being truly the "last resort", and the majority of patients were treated with conservative methods. Another limiting factor in interpreting results in Study II is that a patient receiving surgical treatment had received multimodal treatments before surgery. Hence, comparison of treatments actually is a comparison between conservative treatments with and without surgery and our study setting does not reveal which part of the conservative treatment is actually the most beneficial to the patient. However, in a clinical study setting, this kind of bias is unavoidable because surgery is limited only to the most refractory cases and defined as "the last resort" if other treatments do not relieve the pain sufficiently.

The strengths of Study II are the relatively long-term follow-up (median 36 months) and the measurement of QoL after LPV treatments by using a validated instrument (RAND 36) in a Finnish population. QoL after LPV treatments is an important aspect, bearing in mind the general quality of studies concerning LPV treatments, and the placebo effect (Pereira, 2018). Being single-center investigations, these studies (I and II) brought novel information concerning the efficacy of local (TAUH) treatment protocol and unique patient characteristics that can be used as tools in everyday clinical work. Furthermore, this information can be utilized before surgery of the efficacy of pain reduction and effects on QoL.

The strength of Study III was the assessment of a new family of receptors (ERRs) previously unstudied in association with LPV. Study III also added to the body of evidence concerning other steroid receptors related to LPV, and showing controversial results (Eva et al., 2003; Goetsch et al., 2010; Johannesson et al., 2008; Leclair et al., 2011). Small sample size, unfortunately, prevented any final conclusions being drawn from this analysis.

In relation to Study IV, only few previous report exists concerning possible differences in the vulvar microbiome of LPV patients vs. healthy controls, using the NGS technique(Jayaram et al., 2014). However, the relatively large amount of study patients with unknown day of the menstrual period, 10 out of 30, is a weakness of the study, with possible impact on microbiome. Different age, parity and BMI between study groups can also cause bias when analyzing microbiome. However, these kind of bias in the study population are hard to avoid in the clinical study setting. Further, this kind of cross-sectional study cannot prove causality but

association. The strength of the study was the presentation of novel findings on potential bacteria which may contribute to the development of symptoms, although further and larger studies are warranted to confirm these preliminary findings.

7 SUMMARY AND CONCLUSIONS

The aim of the present study was to clarify certain aspects of vulvodynia, specifically LPV, i.e. etiology, efficacy of different treatment options in a tertiary-center cohort, and patients' QoL. Specific findings in this study were:

1. Combinations of various vulvodynia treatments reduce self-reported pain by half (median NRS scores 8 → 4). Older age (> 30 years) and frequent visits (≥ 6) to outpatient clinics are associated with a smaller reduction in self-reported pain.

2. Surgery, i.e. modified posterior vestibulectomy added to conservative treatments, is more efficient in reducing self-reported pain than conservative treatments assessed immediately after the procedure. However, the two treatment modalities are as efficient regarding self-reported pain and QoL after a median period of 36 months of the follow-up. Conservatively treated LPV patients have lower QoL in certain dimensions compared to healthy women, whereas no differences are found when comparing surgically treated patients to healthy women.

3. ERR β, a member of the orphan nuclear receptor family without an intrinsic ligand, is overexpressed in IHC in vulvar samples from LPV patients compared with healthy controls.

4. The microbiome of the vulvar vestibulum of secondary LPV patients differs from that in healthy controls as regards a total of 31 different bacteria analyzed by NGS. This may reflect the fact that microbial changes in vulvar vestibular flora may contribute to the pathogenesis of secondary LPV, supporting the theory of inflammation or infection as a trigger the pathogenesis of secondary LPV.

8 FUTURE ASPECTS

Multimodal treatments reduce the pain into half from the original. Which part of the multimodal treatment model is the most efficient? In future studies, this is an important issue and resolving this question would help clinicians to direct the limited resources towards the most efficient treatment combination. The role of surgery as a treatment model is another interesting question in the future studies: how to identify the patients to whom surgery is beneficial and who could achieve the same pain relief and QoL with conservative treatments only?

Concerning the etiology of LPV, it has been suggested that hormone receptor signaling could contribute in irritating free nociceptors in the vestibulum by affecting ion channels, which makes them an interesting target for future research in LPV etiopathology. Also, further research of changes in microbiome in LPV is a matter of interest. In the future, clarifying the role of the microbiome in the etiopathology of LPV could lead to possible preventive and therapeutic options in LPV.

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PUBLICATION I

Younger age and combination of therapies associate with significant reduction in self-reported pain

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Combination of treatments with or without surgery in localized provoked vulvodynia - outcomes after three years of follow-up

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Combination of Treatments With or Without Surgery in Localized Provoked Vulvodynia: Outcomes After Three Years of Follow-Up

Anu Pauliina Aalto,[1,2,*] Heini Huhtala,[3] Johanna Mäenpää,[1,4] and Synnöve Staff[1,4]

Most vulvodynia patients receive combinations of several treatment modalities for their chronic painful condition. If conservative treatments fail, vestibulectomy is considered to be the ultimate treatment option for localized provoked vulvodynia (LPV). The aim of this descriptive study was to analyze relief of pain, quality of life (QoL), and complications associated with combining surgery with conservative treatments among LPV patients, both in short term and after 3 years of follow-up.

The study population consisted of a retrospective patient cohort of surgically ($n = 16$) and only conservatively ($n = 50$) treated LPV patients. QoL data were assessed by a validated questionnaire (RAND-36). Data were collected by reviewing patient records and by aid of postal questionnaires. Efficacy of treatments in relief of pain was measured by numerical rating scale (NRS). Two months after surgery, the NRS scores assessed by a physician were lower in the surgery group than in patients treated only conservatively ($p = 0.008$). However, after a median of 36 months of follow-up, self-reported NRS scores and QoL showed no difference between the two patient cohorts. Complication rate after vestibulectomy was 18.8%. The findings suggest that combining surgery with conservative treatments may result in a more effective short-term reduction of pain. However, the effect seemed to be only temporary, as no long-term benefit was achieved.

Keywords: quality of life; RAND-36; vestibulectomy; vulvodynia; vulvodynia treatment

Introduction

Vulvodynia is a chronic pain syndrome of unknown etiology affecting 7–8% of women in population-based epidemiological studies.[1,2] Vulvodynia is usually described as burning, stabbing, itching, stinging, and feeling of irritation. The 2015 Consensus and Terminology and Classification of Persistent Vulvar Pain and Vulvodynia[3] divides vulvar pain into two categories. The first category includes vulvar pain that is caused by a specific clearly identifiable disorder (e.g., pain caused by genital herpes). The second category includes vulvar pain that is at least 3 months in duration and cannot be clearly identified or linked to a specific cause. However, it may have potential associated factors. The descriptors of the pain are location (local, generalized, and mixed), type (provoked, spontaneous, or mixed), onset (primary and secondary), and temporal pattern (intermittent, persistent, constant, immediate, and delayed). Symptoms can overlap and co-occur. Vulvodynia may be associated with a history of yeast infection, hormonal factors, genetic factors, pelvic floor dysfunction, and psychological factors.[3]

The most common clinical subtype of vulvar pain in premenopausal women is localized provoked vulvodynia (LPV).[4] LPV is also considered to be the most common form of sexual pain in women <30 years of age.[5]

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Chronic pain is known to have a negative impact on a woman's quality of life (QoL).[6,7]

Different medical treatment modalities for LPV consist of local, topical, or oral medications. Patients treated by a multidisciplinary team are usually offered physiotherapy (including transcutaneous electrical nerve stimulation), sexual counseling and therapy, and psychotherapy. Although a multidisciplinary approach to LPV is recommended,[8,9] it is actually not evidence based.[10] Surgery (vestibulectomy) for LPV is recommended as the ultimate treatment option, if conservative treatments fail or are insufficient in terms of pain reduction.

Based on studies concerning surgical treatment for LPV, reported success rates vary between 60% and 90%,[11] even though the comparison of different studies is difficult as the term "success," the surgical technique used and the length of follow-up show considerable variation.[11] There is no definitive consensus as to which surgical technique is the superior one. In a review by Tommola et al.,[12] which was based on 33 studies on surgical treatment for LPV (or vulvar vestibulitis), it was concluded that the experience of individual surgeons plays an important role, and that the aim of surgery should be to remove all painful tissues while avoiding unnecessary risks. The review also found surgery to be effective and safe.[12]

Most studies on surgical treatment for LPV lack randomization and/or controls. One of the few randomized controlled studies on vestibulectomy is that by Bergeron et al.,[13] which showed that vestibulectomy was more successful than surface electromyographic feedback and group cognitive-behavioral therapy in pain reduction. As the authors stated, there is a concern in interpreting these results, due to a higher pretreatment drop-out rate in the vestibulectomy group.[13] However, the psychological and sexual functions remained equally positive in all three groups after 6 months of follow-up. Another study that included randomization to the surgical (behavioral treatment and surgery) and nonsurgical (behavioral treatment only) groups, by Weijmar Schultz et al.,[14] found no difference in the outcomes between these two treatment modalities after a mean of 2.5–3 years of follow-up. In the review of Goldstein et al.,[11] surgery was recommended for LPV after failure of conservative treatments (level B evidence).

In previous studies concerning surgery for LPV, the measured outcomes have varied. At least pain reduction,[13,15] dyspareunia,[13,16] sexual functioning,[13] psychological distress,[15] and patient satisfaction[16] have been measured using questionnaires; moreover, findings of physical examination and self-reported symptoms have also been reported. Psychological well-being,[17] quality of sexual life,[17] and sexual and partnership satisfaction have all been reported to improve[18] after vestibulectomy.

The aim of this study was to evaluate the safety and effectiveness of LPV treatments with or without surgery in both short and long terms. Pain was measured by numerical rating scale (NRS) assessed by both a physician and the patient. QoL was evaluated after a combination of treatments with or without surgery, using a questionnaire (RAND-36) validated in the Finnish population.

Materials and Methods

This retrospective cohort study on LPV patients was carried out at the Department of Obstetrics and Gynecology of Tampere University Hospital (TAUH), Tampere, Finland. All at least 18-year-old women diagnosed with vulvodynia at TAUH from January 2003 to May 2016 were screened for the study. Potential vulvodynia patients were identified from the hospital records (computer database) by using the appropriate ICD-10 codes: N90.9 (noninflammatory disorder of vulva and perineum, unspecified); N90.8 (other specified noninflammatory disorders of vulva and perineum); N94.1 (dyspareunia); and N94.2 (vaginismus). Only LPV patients who fulfilled the strict criteria by Friedrich,[19] or severe pain on vestibular touch or attempted vaginal entry, and tenderness on localized pressure within the vulvar vestibule, were considered eligible ($n=66$). Among these eligible patients, 16 patients operated on for LPV (vestibulectomy) were identified. Patients with generalized or continuous vulvar pain were excluded. Other exclusion criteria included malignant tumors of vulva and ongoing inflammatory or dermatological diseases of vulva. The flow chart of study patients is shown in Figure 1.

Information on parity, menopausal status, age, different treatment modalities, and complications after surgery was collected from the hospital's medical records. The baseline pain before any treatments for LPV was assessed by a physician with a cotton swab test and rated on an NRS from 0 to 10. If the rating was not found in the patient record, the information was reported as "no data." As a part of the treatment protocol, every patient had a checkup appointment at 2 months after the surgical treatment with the operating surgeon. Patients treated with conservative methods

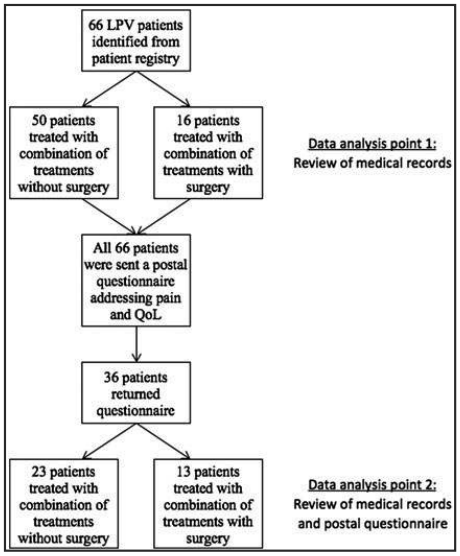

FIG. 1. Patient flow chart.

only were assessed by a physician usually after 2 or 3 months after commencing the treatments. The conservative treatment modalities used for LPV are described in Table 1.

The surgical technique used was the modified posterior vestibulectomy described by Tommola et al.,[16] with the aim to surgically remove the painful vulvar area. The operations were performed under general anesthesia, and all operations were carried out by three senior gynecological surgeons. First, 0.01% lidocain cum adrenalin solution was injected into the vulvar vestibulum for bleeding control and prevention of postoperative pain. To excise vestibular mucosa, 2-mm deep incisions using electrocautery were made from 10 to 2 o'clock in the posterior vulvar vestibulum to a width of ~1–2 cm. The inner incision was made just inside the hymenal ring, and the outer margin followed the Hart's line. The vaginal mucosa was liberated from underlying tissue and subsequently opposed to distal vulvar margin with absorbable sutures without tension.

A seven-page postal questionnaire on demographic data, self-reported pain, and RAND-36 was sent to the 66 eligible LPV patients. The questionnaire was resent to the patients who did not return the questionnaire within 2 months after the first mailing.

The validated Finnish version of the RAND-36-item health survey includes eight multi-item dimensions: general health, physical functioning, mental health, social functioning, vitality, pain, and physical and emotional role functioning.[20,21]

Participants of the study were moreover asked to assess vulvar pain intensity upon touch on the NRS before and after treatments. NRS was used to quantify the intensity of vulvar pain by rating the pain using a 0-to-10 scale, where 0 indicates "no pain" and 10 indicates "the worst pain imaginable."

The study protocol was approved by TAUH Ethical Committee (5APR2016, Identification Code R16053), and a written informed consent was obtained from the patients participating in this study.

Version 23 of IBM SPSS statistics software was used in statistical analyses (IBM SPSS Statistics for Windows, Version 23.0. IBM Corp. 2015. Armonk, NY). Mann–Whitney *U*-test was used for statistical comparisons. A probability value of $p < 0.05$ was considered as statistically significant.

Results

Thirty-six patients (55%) returned the questionnaire during the study period (August 2016–November 2016). Twenty-eight patients returned the questionnaire after the first mailing and eight patients after the second mailing. The patient flow chart is shown in Figure 1. The response rate to postal questionnaires in the nonsurgical group was 46.0% and that in the surgical group was 81.3% ($p = 0.020$). Demographic data and pain before and after the treatments are shown in Table 1. At the data analysis point 1 (2 months after commencing the treatments), the surgical and nonsurgical groups differed significantly in age ($p = 0.048$). The median follow-up time at the data analysis point 2 was 36 months (interquartile range [IQR] = 24–36). The most frequent (received by >50% of the patients) combination of conservative treatments consisted of local treatments (lidocaine and/or gabapentin), physiotherapy, and sexual counseling in both patient cohorts. The treatment modalities used for both patient groups are summarized in Table 1. At the data analysis point 1, the nonsurgical and surgical treatment groups did not differ with respect to any treatment modality. However, at the data analysis point 2, the two treatment groups differed with respect to the frequency of sexual counseling (Table 1; $p = 0.03$).

At data analysis point 1, median pretreatment NRS scores were similar between nonsurgical (i.e., combination of treatments without surgery) and surgical groups

Table 1. Demographic Data and Treatments Given to Localized Provoked Vulvodynia Patients

	Data analysis point 1 (Fig. 1). Review of medical records				Data analysis point 2 (Fig. 1). Review of medical records and postal questionnaire			
	All LPV patients	Combination of treatments without surgery	Combination of treatments with surgery	p[a]	All LPV patients	Combination of treatments without surgery	Combination of treatments with surgery	p[a]
Number of patients	66	50	16	N/A	36	23	13	N/A
Age, median (IQR)	28 (25–33)	27 (24–32.3)	30.5 (26.5–38.3)	0.048	28.5 (25–32)	27 (24–29)	29 (26.5–33)	0.06
Nulliparous, % (*n*)	95.5 (63)	94 (47)	100 (16)	0.32	86 (31)	82.6 (19)	92.3 (12)	0.48
Premenopausal, % (*n*)	98.5 (65)	100 (50)	93.8 (15)	0.08	100 (36)	100 (23)	100 (13)	1.00
NRS before treatments, asked from patients at the time of the cotton-swab test	9 (7.25–9), n.d. $n=22$	9 (7–9), n.d. $n=18$	9 (8–9.5), n.d. $n=4$	0.11	9 (7–9), n.d.=9	8 (7–9), n.d. $n=7$	9 (8–10), n.d.=2	0.014
NRS after treatments, asked from patients at the time of the cotton-swab test	5 (2–8), n.d. $n=24$	7 (4–8), n.d. $n=19$	2 (2–4), n.d. $n=5$	0.008	5 (2–7), n.d. $n=10$	7 (4.5–8), n.d.=7	2 (2–4), n.d.=3	0.005
Self-reported NRS before treatments in the postal questionnaire	N/A	N/A	N/A	N/A	8 (8–9)	8 (7–9)	8 (8–9)	0.66
Self-reported NRS after follow-up in the postal questionnaire	N/A	N/A	N/A	N/A	3 (2–5.75)	4 (3–6)	2 (2–5)	0.18
Treatments received by LPV patients								
Local treatments,[b] % (*n*)	100 (66)	100 (50)	100 (16)	1.00	100 (36)	100 (23)	100.0 (13)	1.00
TCA or anticonvulsant[c]	15.2 (10)	12.0 (6)	25.0 (4)	0.21	16.7 (6)	13.0 (3)	23.1 (3)	0.35
Physiotherapy (including TENS)	90.9 (60)	92.0 (46)	87.5 (14)	0.59	88.9 (32)	91.3 (21)	84.6 (11)	0.46
Sexual counseling by a trained nurse	75.8 (50)	80.0 (40)	62.5 (10)	0.16	77.8 (28)	87.0 (20)	61.5 (8)	**0.03**
Topical treatments[d]	22.7 (15)	18.0 (9)	37.5 (6)	0.11	19.4 (7)	8.7 (2)	38.5 (5)	0.050
Local injections to the painful site[e]	16.7 (11)	16.0 (8)	18.8 (3)	0.80	11.1 (4)	8.7 (2)	15.4 (2)	0.76

[a]p-value between surgical and nonsurgical groups.
[b]Lidocaine gel to the painful area in vulva 30 min before intercourse or gabapentin 6% cream applied twice a day to the painful area for 6–8 weeks.
[c]Amitriptyline 10–40 mg most commonly used TCA or pregabalin 150–300 mg.
[d]Podophyllotoxin (5 mg/mL) applied locally to tender points of vestibulum after 5% acetic acid application. Treated area was covered with a mild estrogen cream and covered with gauze pads until the next day.
[e]2–4 mL of betametasone and long acting anesthetic agent (bupivacaine), both 50% and 50%, injected submucuously to the painful site.
IQR, interquartile range; LPV, localized provoked vulvodynia; N/A, not applicable; n.d., no data; NRS, numerical rating scale; TCA, tricyclic antidepressant; TENS, transcutaneous electrical nerve stimulation.

(median NRS scores 9 in both groups, $p=0.11$, Table 1). Median post-treatment NRS score assessed by a physician in different treatment groups was 7 and 2, respectively ($p=0.008$). After median of 36 months of follow-up, self-reported NRS scores before or after treatments did not differ significantly between the groups ($p=0.66$ and $p=0.18$, respectively, Table 1). At data analysis point 2, we also compared medical record-derived data assessed by a physician. Physician-assessed NRS score before treatment in the nonsurgical group was 8 and that in the surgical group was 9 ($p=0.014$). Similarly, post-treatment NRS score assessed by a physician was 7 and 2, respectively ($p=0.005$). Among the LPV patients who did not respond to postal questionnaires ($n=30$), the median pretreatment NRS score collected from the patient records was 9 (IQR=8–9.5, missing data $n=13$), and the median 2-month post-treatment NRS score was 5 (IQR=2.25–8, missing data $n=14$). When nonresponders were compared with all LPV patients who returned the questionnaire (data analysis point 2), the pre- and post-treatment NRS scores derived from the medical records were similar ($p=0.291$, $p=0.592$, respectively).

The QoL after a median of 36 months of follow-up after treatments did not differ significantly between the surgical and nonsurgical groups in any of the eight multi-item dimensions (Table 2 and Fig. 2).

Out of 16 patients operated on, 3 had complications after surgery, resulting in a complication rate of 18.8%. One patient had heavy postoperative pain and

Table 2. Quality of Life After Follow-Up in Different RAND-36 Dimensions

	Combination of treatments with surgery	Combination of treatments without surgery	p^a
Physical functioning/ health, mean (SD)	95.4 (15.20)	92.4 (14.45)	0.243
Physical role functioning, mean (SD)	84.6 (33.13)	69.6 (43.92)	0.278
Emotional role functioning, mean (SD)	66.7 (40.82)	56.5 (46.53)	0.498
Vitality, mean (SD)	58.1 (16.65)	51.5 (23.95)	0.518
General mental health, mean (SD)	68.9 (22.87)	65.7 (21.77)	0.416
Social functioning, mean (SD)	79.8 (19.46)	72.3 (27.94)	0.485
Pain, (SD)	75.2 (26.76)	64.7 (24.50)	0.144
General health perceptions, mean (SD)	63.9 (21.03)	62.2 (23.88)	0.974

[a]*p*-value between surgical and nonsurgical treatment groups.
SD, standard deviation.

was readmitted to hospital on the third postoperative day. Two months after surgery, the patient was still suffering from pain, whereas after 1 year of follow-up the pain in the vulvar area was "transformed into a neuropathic pain," and the patient was treated with peroral gabapentin, which resulted in sufficient pain relief. Another patient was readmitted after 7 days of surgery, because of a partial wound dehiscence. The wound was reported to have healed completely at the 2-month follow-up visit. The third patient suffered from severe pain right after surgery, and had to stay overnight at the hospital. At 2-month follow-up, the pain score was assessed as "0" by the operating physician.

Discussion

We describe here a retrospective cohort of 66 LPV patients treated at our institution. We evaluated short-term surgical complications, pain, and QoL of nonsurgically and surgically treated patients first after 2 months and then after a median of 36 months of follow-up. QoL after 36 months did not differ when comparing the surgically and only conservatively treated groups in any of the eight QoL dimensions of validated RAND-36 questionnaire. Addition of surgery to the conservative treatments resulted in lower NRS scores measured by a physician 2 months after surgery. However, there was no difference in self-reported NRS pain scores measured after the longer follow-up.

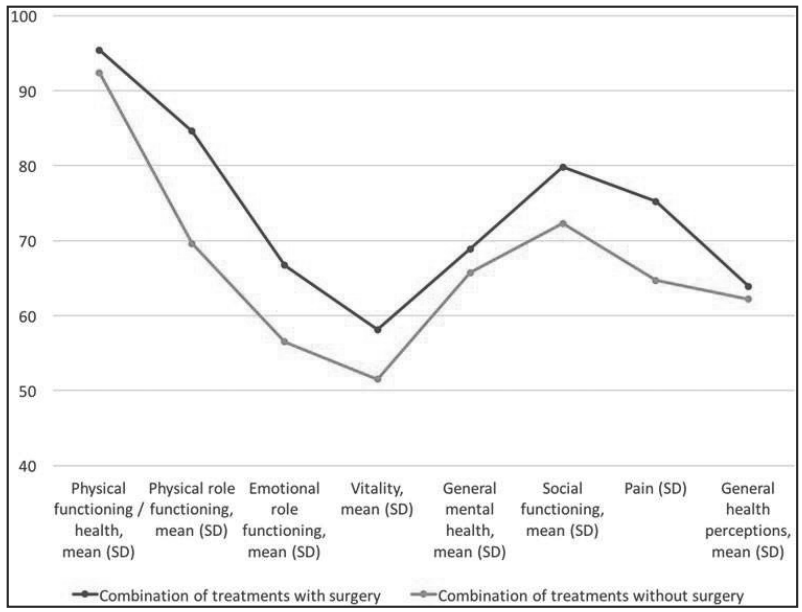

FIG. 2. Quality of life of LPV patients measured with RAND-36. LPV, localized provoked vulvodynia.

Vestibulectomy seems to be a safe treatment modality for LPV with an acceptable complication rate. This is in line with the previous review by Tommola et al. concerning surgery for LPV.[12] In this study, surgery was associated with better short-term outcomes in terms of pain after 2 months of surgery. Previously, it has been shown that median pain measured with VAS decreases from 8 to 2 in surgically treated patients,[18] which is of a magnitude similar to our results. However, assessment of pain at the checkup visits shortly or at any time point after surgery by the attending surgeon is not blinded and certainly at risk of many types of bias. This bias may also explain the differences shown here between NRS values obtained from medical records and those reported by patients themselves. In a randomized study,[14] surgical intervention added to behavioral approach had outcome similar to behavioral approach after 2.5–3 years of follow-up. A similar outcome was found among patients given an opportunity to choose between surgery and no surgery. Although the sample size in the study was small ($n=14$ in the randomized part of the study), it being a randomized study strengthens the perception that individual tailoring of treatment is one of the key factors in a successful treatment for LPV.

We report here QoL data obtained with a validated questionnaire among surgically and nonsurgically treated vulvodynia patients. There was no difference in QoL between these two patient groups after a median of 3 years of follow-up. Previously, Bohm-Starke and Rylander have reported that vestibulectomy improves QoL measured by VAS from median 0.5 to 6.5, during a median 41 months of follow-up.[17] However, another long-term follow-up study on LPV patients treated conservatively versus treated surgically failed to show any difference in long-term well-being between the treatment groups.[18] Even if there are previous valuable reports on QoL and overall well-being among vestibulectomy patients,[17,18] to our knowledge this is the first report using a validated QoL questionnaire when assessing QoL among vestibulectomy patients.

There are some limitations to our study. The study is a retrospective nonrandomized cross-sectional study. An ideal study setting would have been a comparison between only surgically and nonsurgically treated patients preferably as a randomized controlled study. A confounding factor is that the study patients in the surgical group had also received various conservative treatments before surgery, that is, the comparison between the groups is in fact a comparison between combination of treatments with and without surgery. Because both groups received various conservative treatment modalities it is not possible to conclude fully the effectiveness of surgery. However, this setting is clinically unavoidable since vestibulectomy is the treatment modality offered to patients only after failure of all noninvasive treatments. The fact that patients were asked to report pain retrospectively after a median of 36 months after treatments contains also a risk of bias. However, the median follow-up time after treatments did not differ significantly between surgically and nonsurgically treated patients ($p=0.35$) and QoL measured corresponded to present moment (i.e., the time of questionnaire). A longitudinal QoL evaluation, done before and after treatments, would have been of additional value.

Although the total number of patients is relatively low, with the surgically treated group being even smaller, it reflects the fact that vulvodynia is a rather rare condition. The response rate after follow-up was only satisfactory, 55%. The response rate to postal questionnaires of surgically treated patients was higher and this may lead to a false accentuation of positive effect of the intervention, that is, to a type I error. However, pain rated on the NRS and QoL did not differ significantly between the groups after the longer follow-up. The amount of missing data was unfortunately also quite high and this may cause bias. The study patients had received slightly different conservative treatment entities before surgery or during the treatment period that might have an effect on outcome, too.

Conclusion

Measuring QoL with a validated questionnaire in the Finnish population can be considered as strength of the study. Bearing in mind the limitations, as discussed earlier, we conclude that even if surgery seems to be associated with more effective pain management in the short term, it showed no additional benefit with respect to QoL or pain after extended follow-up. In contrast, it may be concluded that performing vestibulectomy after conservative treatments is safe and does not seem to be harmful. However, long-term patient-reported outcomes in terms of QoL and pain after surgery do not seem to differ from those achieved conservatively. Considering recent evidence of a strong placebo effect concerning medical treatments,[22] prospective sufficiently powered controlled trials are truly warranted.

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Author Disclosure Statement

The authors (A.P.A., J.M., and S.S.) report conflict of interests. Authors (H.H.) report no conflict of interests. A.P.A. has received a congress travel grant from Roche. J.M. has received congress travel grant from AstraZeneca and Roche and has worked as a consultant for AstraZeneca, Tesaro, Roche, MSD, and Clovis. S.S. has received congress travel grants from Roche, Astra Zeneca, and Tesaro.

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Abbreviations Used

IQR = interquartile range
LPV = local provoked vulvodynia
N/A = not applicable
n.d. = no data
NRS = numerical rating scale
QoL = quality of life
TAUH = Tampere University Hospital
TCA = tricyclic antidepressant
TENS = transcutaneous electrical nerve stimulation
VAS = visual analogue scale

PUBLICATION III

Expression of estrogen-related receptors in localized provoked vulvodynia

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Expression of Estrogen-Related Receptors in Localized Provoked Vulvodynia

Anu Aalto,[1,2,*] Riitta Huotari-Orava,[1,3] Satu Luhtala,[1] Johanna Mäenpää,[1,4] and Synnöve Staff[1,4]

Abstract

Eight percent of women suffer from vulvodynia, a chronic pain condition with unknown etiology. Inflammation and dysregulation of estrogen signaling have been suggested to play a role in the pathogenesis of localized provoked vulvodynia (LPV). Therefore, the aim of the study was to analyze protein expression levels of estrogen-related receptors ERRα, ERRß, ERRγ, estrogen receptor (ERα), and progesterone receptor (PRα) and CD3-positive T cells in the vulvar vestibulum obtained from women suffering from LPV in comparison to healthy, unaffected controls. Vulvar vestibulum tissue specimens were obtained from LPV patients ($n = 12$) who had undergone modified posterior vestibulectomy and from 15 healthy controls. Protein expression of ERRα, ERRß, ERRγ, ERα, and PRα and CD3-positive T cells was analyzed by immunohistochemistry (IHC). Expression of ERRß was significantly more pronounced in samples from LPV compared to healthy controls ($p = 0.006$). No significant difference in the expression patterns of ERRα, ERRγ, ERα, PRα, or CD3 cells was detected. To our knowledge, this is the first study reporting ERR expression in normal vestibulum and in vestibulectomy samples from LPV patients. The higher level of ERRß expression detected by IHC may reflect dysregulation of estrogen signaling in LPV.

Keywords: estrogen-related receptor; estrogen receptor; progesterone receptor; vulvodynia; localized provoked vulvodynia; vulvodynia etiology

Introduction

Approximately 8% of 18–70-year-old women are estimated to suffer from vulvodynia, a chronic pelvic and vulvar pain condition of unknown origin.[1] Localized provoked vulvodynia (LPV) is considered to be the most common form of sexual pain in women younger than 30 years of age.[2] The etiology of LPV is multifactorial and remains mostly unknown.

The role of hormone signaling[3–5] and inflammation[6] in LPV has previously been addressed in few studies. It has been suggested that estrogen receptor (ER)α is expressed at significantly higher level in LPV samples compared to healthy controls, while no difference in the expression of ERß and progesterone receptor (PR) A or B was detected.[3] The findings concerning ERα, PR A, and B expression have been contradictory when samples of primary and secondary LPV have been compared.[4,5]

Similarly, studies reporting the amount of T cells in LPV specimens have been inconsistent.[5–8] However, there is evidence of deregulated inflammation in LPV since proinflammatory mediators such as tumor necrosis factor-a and interleukin1-b have been shown to be elevated in women with LPV.[6] In addition, greater numbers of B lymphocytes and mature mucosal IgA-plasma cells with a difference in B and T cell arrangement in germinal centers have been detected in vulvar vestibulum of LPV women.[8]

The estrogen-related receptors (ERRs) are a small family of orphan nuclear receptor transcription factors

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that are not yet associated with a natural ligand and therefore considered as orphan receptors.[9] The ERRs were originally discovered due to their similarity to the ERs, however, they do not bind to estrogen itself.[9] ERR family include three isoforms, ERRα, ERRβ, and ERRγ (American nomenclature committee NR3B1, NR3B2, NR3B3, respectively).[10]

Sequence alignment of ER and ERRα is highly similar; 68% in the DNA-binding domain and moderately similar (36%) in the ligand-binding E domain.[11] The ERRs are essential factors for normal mitochondrial function.[12] They also affect many cellular processes by regulating cell energy metabolism, immune response through T cell activation and differentiation, and participate in the estrogen signaling pathway.[13] High levels of ERRα is found in tissues with high metabolic needs, such as intestinal tract, heart, kidneys, and skeletal muscle.[12] ERRβ and ERRγ are also expressed in heart and kidney and all three isoforms are expressed in the central nervous system.[12]

Expression of ERRs has been previously studied in benign (lichen sclerosus et atrophicus, LSA), precancerous, and malign vulvar epithelium.[14] However, previous data are available on ERR isoform expression in vulvar vestibulum neither of healthy women (i.e., no dermatological or pain problems) nor of women suffering from LPV.

The hormonal and inflammatory factors may also be interrelated in the pathogenesis of LPV, since estrogen has been shown to modulate immune response by restricting neutrophil accumulation to the site of inflammation, attenuating the release of proinflammatory mediators and regulating ER gene expression in T-, B-, and dendritic immune cells.[15] In addition, a wide range of rapid estrogenic actions have been shown on different tissues and cell types by modulating the permeability of different ion channel types[16] and thereby influencing immune response.[17]

Hormone signaling has been shown to act through transient receptor potential (TRP) channels affecting nociceptor excitability and sensitization in many chronic pain syndromes.[18] Inflammation is also one possible trigger in peripheral sensitization,[17] which typically occurs in LPV. Inflammatory mediators can cause an increase in the excitability of peripheral nociceptors[17] in LPV. Therefore, estrogen signaling could theoretically be actionable in LPV by affecting ion channel permeability, which contributes to nociceptor excitability and sensitization.

Therefore, the aim of this study was to analyze the expression of all three ERR isoforms by immunohistochemistry (IHC) from LPV samples in comparison with samples from healthy women. In addition, ER and PR expression was also studied, and the state of inflammation was also addressed by assessing the total amount of T cells by analyzing the staining of CD3-positive antibody.

Materials and Methods

This study on LPV patients was carried out at the Department of Obstetrics and Gynecology of Tampere University Hospital (TAUH), Tampere, Finland and the Department of Obstetrics and Gynecology in Kanta-Häme Central Hospital (Hämeenlinna, Finland). The study protocol was approved by TAUH Ethics Committee (5APR2016, R16053), and a written informed consent was obtained from all the healthy controls who volunteered in this study. The Finnish National Supervisory Authority for Welfare and Health gave its permission to use the archival vestibulectomy samples in the present study without consulting the patients.

Twelve modified posterior vestibulectomy samples were collected from the hospital archives (all vestibulectomies performed in TAUH between January 2003 and May 2016). All patients operated had been diagnosed with LPV before surgery according to Friedrich's criteria,[19] that is, severe pain on vestibular touch or attempted vaginal entry and tenderness on localized pressure within the vulvar vestibule. All patients had received conservative treatments in different combinations for their LPV before operation. The macroscopic and morphological findings of the vestibulectomy specimens were confirmed by an experienced pathologist as a part of routine diagnostics in TAUH department of pathology. Patients with vulvar malignancy, ongoing inflammatory, or skin diseases of vulva were excluded from this study.

As healthy controls, we prospectively recruited 15 healthy volunteers aged 18–40 from Kanta-Häme Central Hospital and TAUH. The exclusion criteria were pregnancy, history of vulvar malignancy, any inflammatory, or skin disease of any part of the body and any type of localized or generalized pain syndrome. Healthy controls were admitted to hysteroscopy for benign reasons (generally hypermenorrhea with a polyp or a fibroid) under general anesthesia or as an office procedure. We used local anesthetic agents (1–2 mL of 0.01% lidocain with adrenalin), and 6 mm punch biopsy from vulvar vestibulum at 7 o'clock was taken. Punch biopsies were routinely embedded in paraffin after a maximum of 24 h of fixation in 10% buffered

formalin. All the control biopsies were taken at a standardized time point of menstrual cycle (before cycle day 12).

The demographic data on vulvodynia patients were collected from the hospital register (age, parity, menopausal status, different treatments given before vestibulectomy, and medication). For healthy controls, a short questionnaire containing demographic data, current medication, the phase of the menstrual cycle for the study purposes was filled by a physician at the time of the punch biopsy.

All IHC stainings were performed in the Tampere Histology Facility (HF) at Tampere University. For IHC stainings, formalin-fixed and paraffin-embedded biopsies were routinely processed and cut into 4–5 μm thick serial sections, baked and deparaffinized with n-hexane. Before IHC, standard hematoxylin and eosin (H&E) staining was performed. Before immunostaining, antigen retrieval was done by boiling the slides in TE buffer (50 mM Tris-HCl, 1 mM EDTA pH 9) at +121°C for 2 min. Endogenous peroxidase was blocked by incubating the slides with 3% H_2O_2 for 5 min. ER- and PR-stainings were performed with mouse monoclonal antibodies, clones 6F11 (ER) and PGR-312 (PR), both diluted at 1:200 (Leica BioSystems Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK). For CD3 staining, we used anti-CD3e rabbit monoclonal antibody detecting both CD4- and CD8-positive T cells (clone BSR10) at a dilution of 1:200 (Nordic BioSite Ab, Täby, Sweden). IHC- for ERR were performed with mouse monoclonal anti-human ERRα (clone H5844), ERRβ (clone H6705), and ERR-γ (clone H6812) antibodies (Perseus Proteomics, Inc., Tokyo, Japan) that were diluted at 1:500, 1:50, and 1:50, respectively. Sample slides were incubated with primary antibodies for 30 min at room temperature. For the detection, Histofine® Simple Stain MAX PO Multi HRP polymer and Histofine DAB-2V kit (both from Nichirei Biosciences, Inc., Tokyo, Japan) were used according to the manufacturer's instructions. Samples were counterstained with Mayer's hematoxylin with addition of 0.5% $CuSO_4$ to intensify the DAB reaction. All the IHC stainings were conducted with an Autostainer 480S immunostainer (Lab Vision Corporation, Fremont, CA). Histologically normal skin and colon tissues were used as positive control samples in the IHC-stainings and were provided from the archives of TAUH Pathology department.

All the stainings were evaluated by an experienced dermatopathologist (R.H.O.) and the first author (A.A.). Immunohistological sections were analyzed under a light microscope (Olympus BX51, Model U-MDOB3, Tokyo, Japan) from representative areas. Staining for ER and PR were scored similar to routine breast pathology using a 0–3 scale: 0 = negative; 1 = <10%; 2 = 11–50%; 3 = 51–100% (×20 objective).[20]

Stainings of ERR-receptors were graded using a scale of 0/+/++/+++ (+++ = increased staining compared to control, ++ = stained as control, + = decreased staining compared to control, 0 = unstained/negative) (×20 objective). CD3-positive T cells were analyzed by counting the mean number of positive cells per field from 2 to 4 high power fields (hpfs) (×40 objective). CD3-cells were graded as 1 ≤ 50 cells/hpf, 2 = 50–100 cells/hpf, and 3 ≥ 100 cells/hpf. The scoring of each section was based on a consensus of two investigators and possible disagreements were resolved by a joint review.

Version 24 of IBM SPSS statistics software was used in statistical analyses (IBM SPSS Statistics for Windows, Version 24.0; IBM Corp. 2016. Armonk, NY). A Fisher's exact test and Mann–Whitney *U*-test were used for statistical comparisons when appropriate. A probability value of $p < 0.05$ was considered as statistically significant.

Results

Patient demographics are shown in Table 1. The groups were similar with regard to menopausal status and the use of combined and progestin only contraceptives. Healthy controls were older than LPV patients (median age of 39 vs. 27 years, respectively, $p = 0.016$). Also, LPV patients were more often nulliparous than healthy controls (83.3% vs. 40%, respectively, $p = 0.047$).

The H&E stainings from all the LPV patients in comparison to two healthy controls are shown in Figure 1. No specific pathological diagnostic abnormality was detected in LPV patients or in healthy controls. Chronic

Table 1. Patient Characteristics of Localized Provoked Vulvodynia Patients and Healthy Controls

	LPV patients (*n* = 12)	Healthy controls (*n* = 15)	*p*
Age, median (IQR)	27 (23.25–34.75)	39 (34–44)	0.016
Premenopausal, *n* (%)	11 (91.7)	15 (100)	0.44
Nullipara, *n* (%)	10 (83.3)	6 (40.0)	0.047
Combined contraceptives, *n* (%)	1 (8.3)	2 (13.3)	1.00
Progestin only, *n* (%)	2 (16.7)	1 (6.7)	0.57
Symptom duration in months, median (IQR)	20.5 (12–23.5)	n/a	n/a

IQR, interquartile range; LPV, localized provoked vulvodynia; n/a, not applicable.

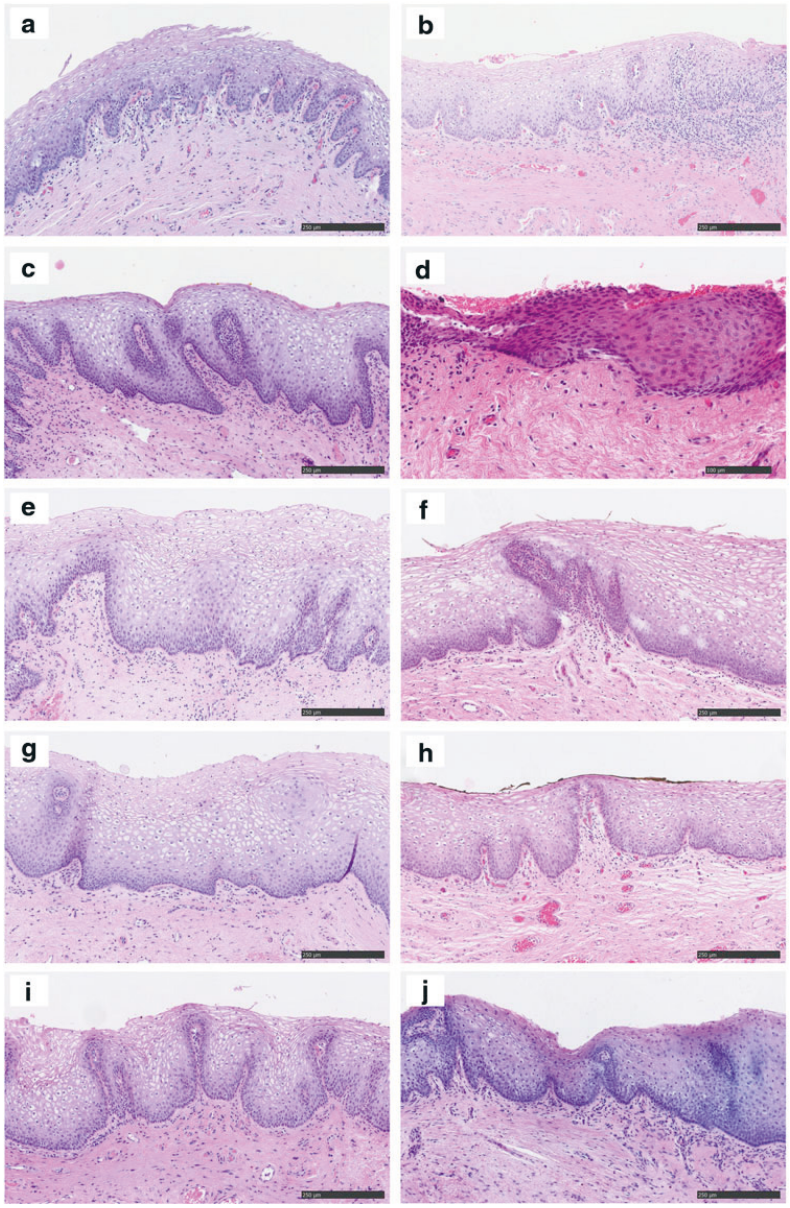

FIG. 1. Hematoxylin and eosin stainings of the LPV patients **(a–l)** and examples of two healthy controls **(m, n)**. All the histologic sections were visualized with the 10/20 × objective. LPV, localized provoked vulvodynia.

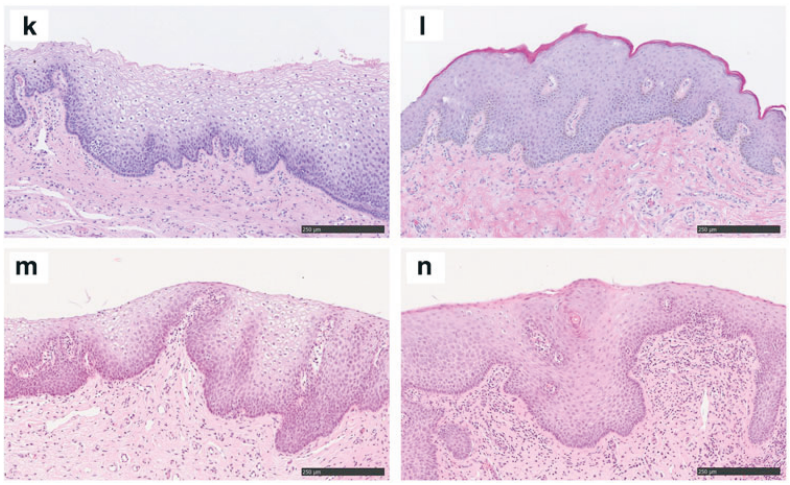

FIG. 1. (Continued).

nonspecific inflammation was detected in three samples from LPV women (Fig. 1b, f, i). The normal vestibulum from control patients showed uniform staining pattern of all ERR isoforms analyzed. Both in the study and control samples, ERRα and ERRß expression was both nuclear and cytoplasmic, while ERRγ showed only nuclear staining by IHC (Fig. 2). Overall, ERRß staining (both nuclear and cytoplasmic) was statistically significantly more pronounced in LPV samples compared to healthy controls (Fig. 2b1, b2, Table 2, $p = 0.006$). No difference was found in the level ERRα and ERRγ expression (Fig. 2a1, a2, c1, c2, Table 2). Staining of ER, PR, and CD3 was also similar between LPV and control patients (Fig. 2d1–f2, Table 2).

Discussion

We report in this study the expression of ERR isoforms in vulvar vestibulum of LPV patients compared to healthy controls. To our knowledge this is the first study to report the expression of ERRs in relationship to LPV. We also describe the expression of ERRs in the normal vulvar epithelium by IHC. We report here that ERRß expression was more pronounced in the vulva of LPV patients compared to healthy controls. We also report no difference in the expression of ERRα and ERRγ in LPV samples.

We have showed here that normal vulvar vestibulum expressed all ERR isoforms uniformly. Only one previous study has demonstrated the expression of ERR isoforms in normal vulvar skin, but the control/normal population consisted of LSA patients providing control biopsies from normal appearing skin in the vulvar area.[14] Our findings are consistent with that previous study, but our control samples represent more adequately normal healthy vulvar epithelium since control patients with any history of skin diseases were excluded. There are only few studies addressing ERR expression in normal genital organs such as vagina or endometrium.[21,22]

Previously, Cavallini et al. have described the mRNA expression of ERRs in the premenopausal and postmenopausal human vagina.[21] They showed a significant decline in ERRα and ERRγ expression in the vaginal epithelium in postmenopausal women, but this was not clearly observed for ERRβ.[21] ERRβ expression has also been studied in normal human endometrium.[22] ERRβ mRNA and protein were expressed in healthy human endometrium, but ERRβ protein was mainly localized in the nuclei of both stromal and endometrial cells.[22] In this study, in contrast, we show that ERRβ was expressed uniformly both in the cytoplasm and nucleus in healthy vulvar epithelium. This may reflect a difference in the functions of ERRβ between normal endometrium and vulva and warrants further investigations.

The expression of ERR isoforms has also been evaluated in certain gynecological disease conditions, but

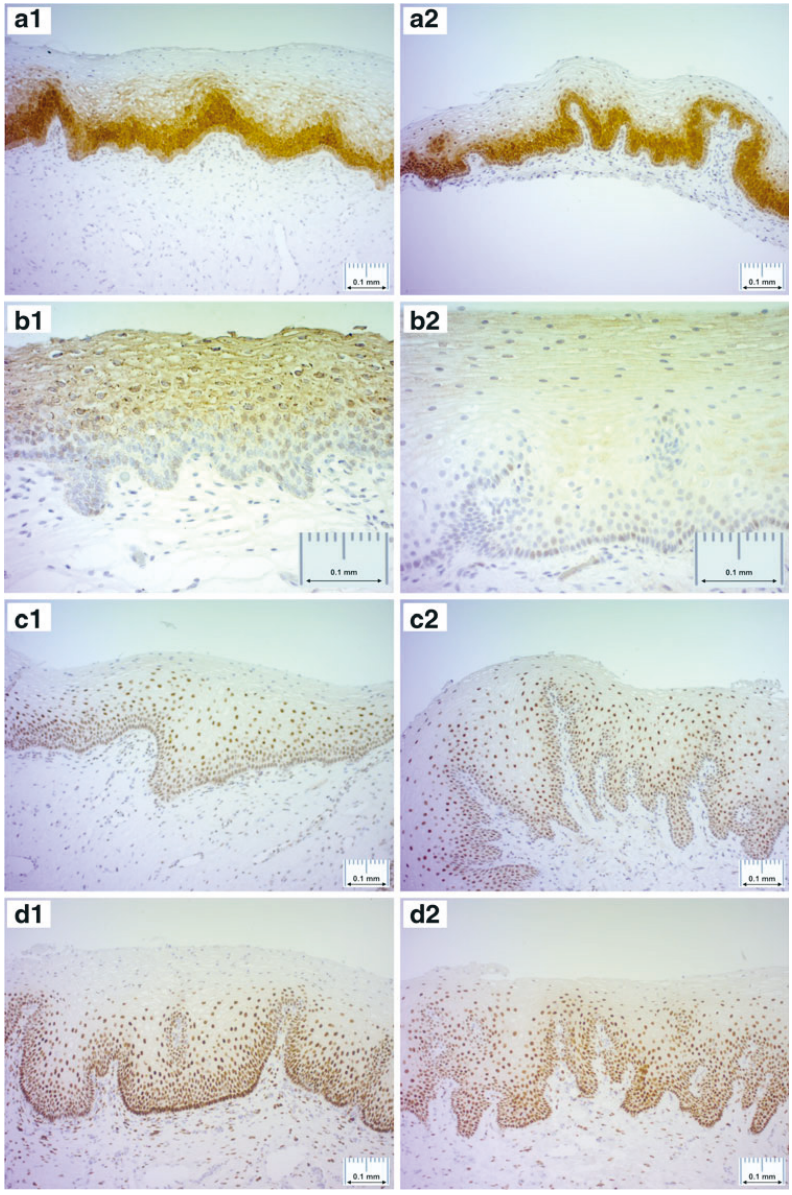

FIG. 2. Examples of ERRα immunostaining showing similar cytoplasmic and nuclear staining in vulvar vestibulum from LPV patients **(a1)** and healthy controls **(a2)**. Examples of ERRß immunostaining showing more intense cytoplasmic and nuclear staining in vulvar vestibulum samples from patients with LPV **(b1)** compared to samples from healthy controls **(b2)**. Examples of ERRγ immunostaining demonstrating similar nuclear staining in vulvar vestibulum from LPV patients **(c1)** and healthy controls **(c2)**. Examples of ER immunostaining showing similar expression pattern between samples from LPV patients **(d1)** and healthy controls **(d2)**. Examples of PR immunostaining in vulvar vestibulum samples from LPV patients **(e1)** and healthy controls **(e2)**. Examples of CD3 immunostaining identifying both CD4- and CD8-positive T cells in vulvar vestibulum samples from patients with LPV **(f1)** and healthy controls **(f2)**. All the histologic sections were visualized with the 10/20$\times$objective using an Olympus BX-51 light microscope (Tokyo, Japan). ER, estrogen receptor; ERR, estrogen-related receptor; PR, progesterone receptor.

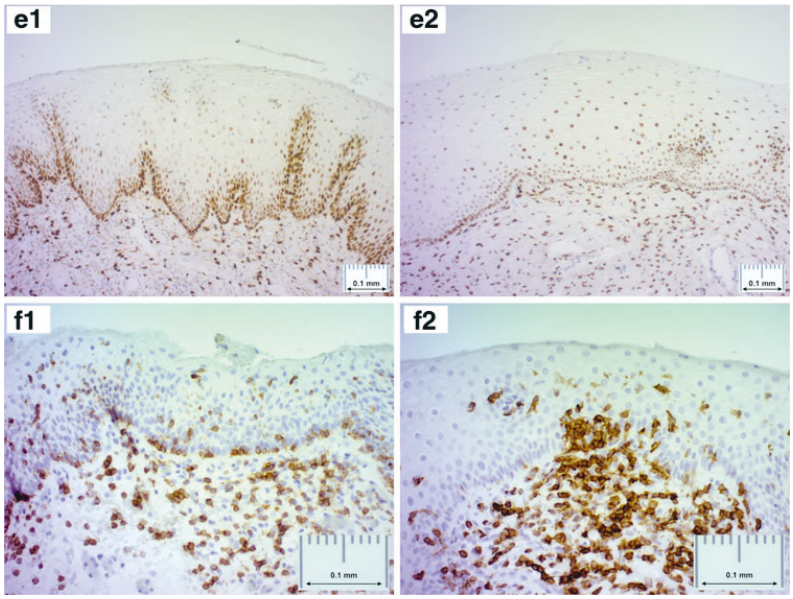

FIG. 2. (Continued).

the data reported here regarding expression of ERRs in LPV are novel. ERRα has been shown to decrease in the pathogenesis of vulvar cancer in LSA-positive background.[14] In contrast, ERRα mRNA was shown to be upregulated in ovarian cancer, while the levels of ERRβ and ERRγ were undetectably low.[23] The expression of ERRα and ERRγ has also been shown to decrease in endometriotic lesions, but this was not seen in the case of ERRβ.[24] In endometrial cancer, increased expression of ERRα has been associated with advanced clinical stage and aggressive histology and ERRα silencing resulted in reduced cell proliferation *in vitro*.[25] All these data imply that dysregulation of ERRs may be active in various gynecological disease conditions.

We have shown here that ERRβ was expressed at higher levels in LPV. The differential expression of ERRβ was not related to differential expression of ER, PR, or to the amount of CD3-positive T cells. In our material, the staining patterns of ER, PR, and the amount of T cells were similar between the study and control samples. Few studies have concentrated on the expression of steroid receptors in LPV. The ER expression in vulvar vestibulum of LPV patients has been previously shown to be both up- and downregulated.[3–5] The contradictory results obtained may be partly explained by differences in the used study methodology and classification. In this study, we have used the standardized methodology validated in breast cancer diagnostics.[20] Our finding of similar expression of PR and CD3-positive T cells is consistent with previous studies showing no difference between LPV and control samples.[3,8]

ERRs are known to have an impact on estrogen signaling pathways and immunology, which are both possible etiological factors contributing to the pathogenesis of LPV.[6,15] Finding that ERRβ is significantly more pronounced in LPV patients' samples may reflect the role of dysregulation of estrogen signaling in LPV. ERRβ has several splice variants and its functions have not been so well understood compared to other ERR isoforms.[26]

Estrogen and sex hormones in general have been shown to play a role in peripheral sensitization by regulating the permeability of different ion channels such as potassium and calcium channels.[17] Sex hormones have been shown to act through the superfamily of TRP channels, which can act as molecular sensors of chemical and physical stimuli.[18] Activation of TRP channels in nociceptors result in complex intracellular

Table 2. Stainings of Estrogen Receptor α, Progesterone Receptor α, CD3, ERRα, ERRß, and ERRγ

	LPV patients ($n=12$)[a]	Healthy controls ($n=15$)[b]	*p*
ER			
Not stained	0	0	0.181
<10%	0	0	
11–50%	0	2	
51–100%	12	12	
PR			
Not stained	0	0	0.078
<10%	1	5	
11–50%	3	4	
51–100%	8	5	
CD3			
<50 cells/hpf	0	0	0.236
50–100 cells/hpf	1	4	
>100 cells/hpf	10	10	
ERR α			
Stained less than controls	0	0	1.00
Stained as controls	11	14	
Stained more than controls	0	0	
ERR ß			
Stained less than controls	0	0	0.006
Stained as controls	6	14	
Stained more than controls	5	0	
ERR γ			
Stained less than controls	0	0	1.00
Stained as controls	11	14	

[a]One vestibulectomy sample was sufficient only for ER and PR stainings.
[b]One of the control samples taken did not contain epithelium, sample was excluded from the analysis.
ER, estrogen receptor; ERR, estrogen-related receptor; hpf, high-power field; PR, progesterone receptor.

signaling cascade leading to either neuronal adaptation, that is, desensitization or potentiation.[27,28] In theory, the present finding of high expression of ERRβ in LPV may suggest that this mechanism can be in actionable also in LPV. However, such conclusion cannot be made from the present data but confirmatory extension studies are needed to resolve the possible role of estrogen signaling in the pathogenesis in LPV.

Limitations of our study include a limited sample size. However, other articles reporting staining patterns of vestibulectomy samples have also been quite limited with respect to sample size.[3,5] This is mostly due to the relative rarity of the disease and especially the surgical treatment. The study cohort was retrospective generating possible bias with the collection of demographic data. However, the control samples were collected prospectively and it may be regarded as strength of the study. Therefore, the control group consisted only of women who had never suffered from any dermatological or pain problems. In addition, the phase of the menstrual cycle could be standardized in the control group. The study and control groups did not differ with respect to hormonal contraceptive use, which is of importance when studying expression of hormonal factors.

Conclusions

ERRs are involved in estrogen signaling and in many essential cellular functions. We have shown here that ERRβ expression was increased in vulvar vestibulum from LPV patients. This finding regarding ERRβ expression in LPV warrants further validation in larger, independent LPV cohorts. If validated, ERRβ may serve as a possible target for the future treatments of LPV.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

DAB = 3,3′-Diaminobenzidine
EDTA = ethylenediaminetetraacetic acid
ER = estrogen receptor
ERR = estrogen-related receptor
H&E = hematoxylin and eosin
HF = Histology Facility
hpf = high power field
IHC = immunohistochemistry
IQR = interquartile range
LPV = localized provoked vulvodynia
LSA = lichen sclerosus et atrophicus
PR = progesterone receptor
TAUH = Tampere University Hospital
TRP = transient receptor potential

PUBLICATION IV

Secondary vulvodynia patients show reduced bacterial diversity in vestibular microbiome.

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(Submitted)