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**COMBINED ORAL
CONTRACEPTIVES - IMPACT
ON THE VULVAR VESTIBULAR
MUCOSA AND PAIN
MECHANISMS**

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ABSTRACT

Objective: The main aim of this thesis was to study the impact of combined oral contraceptives (COC) on the vulvar vestibular mucosa and pain mechanisms in healthy women and in women with provoked vestibulodynia (former vulvar vestibulitis syndrome). The somatosensory perception in the vulvar vestibular mucosa of healthy women was studied with the relation to COC. An endogenous pain inhibitory response called diffuse noxious inhibitory controls (DNIC) was examined in healthy women with or without COC and in women with provoked vestibulodynia. The morphology and steroid receptor expression in healthy women during the influence of COC and during the menstrual cycle and in women with provoked vestibulodynia was evaluated.

Material and Methods: Thirty four women with provoked vestibulodynia, 60 healthy women using COC and 64 healthy non COC users participated in the studies. Quantitative sensory tests, including mechanical and thermal pain thresholds of the vulvar vestibule were performed. Pressure pain thresholds (PPTs) were measured before and during a cold pressor test to provoke a DNIC, or “pain inhibits pain” response. Vestibular biopsies were collected for morphological analyses. The amount and distribution of estrogen receptors α and β , progesterone receptors A and B, glucocorticoid receptor, androgen receptor and the proliferation marker Ki67 were estimated using immunohistochemistry followed by computerized image analysis.

Results: The mechanical pain thresholds were significantly lower in women using COC than in nonusers. An intact DNIC response was present in all three groups as illustrated by a significantly increased PPT during cold noxious stimulation. Compared with the healthy women, the patients displayed lower PPTs, both before and during the cold pressor test. The vulvar vestibular mucosa displayed a larger interdermal papilla distance in the luteal phase compared with the follicular phase. A similar morphological feature was seen in COC users and there was also a larger distance from the dermal papillae to the epithelial surface compared with controls. Histopathological assessments showed a higher amount of superficial blood vessels in the COC users. The vestibular stromal tissue expressed more ER β in women with COC than in women without. PRB was more abundant in the stromal tissue in the follicular phase than in the luteal phase. There was a significantly higher expression of ER α in both the epithelium and the stroma in the specimens of the vestibulodynia patients compared with that of controls.

Conclusions: COC may induce an increased sensitivity in the vestibular mucosa in healthy women and might be one contributing factor in the development of provoked vestibulodynia. An altered morphological pattern and changes in the expression of various hormone receptors in women using COC and during the luteal phase indicates a gestagenic effect on the mucosa. There is a systemic hypersensitivity in women with provoked vestibulodynia; however the endogenous pain inhibition seems comparable to that of healthy women irrespective of COC use. The increased expression of ER α in women with provoked vestibulodynia, without an effect on the epithelial morphology, may be related to an ongoing neurogenic inflammation in the mucosa.

Key words: combined oral contraceptives, quantitative sensory testing, pain thresholds, DNIC, endogenous pain modulation, provoked vestibulodynia, vulvar vestibulitis syndrome, morphology, vulvar mucosa, steroid receptors

To Joacim, Fabian and Disa with love ♥

LIST OF PUBLICATIONS

This thesis is based on the following papers and manuscripts, which will be referred to in the text by their Roman numerals (I-V).

- I. Bohm-Starke N, **Johannesson U**, Hilliges M, Rylander E, Torebjork E. Decreased mechanical pain threshold in the vestibular mucosa of women using oral contraceptives: a contributing factor in vulvar vestibulitis? *Journal of Reproductive Medicine*. 2004;11:888-92.
- II. **Johannesson U**, de Boussard CN, Brodda-Jansen G, Bohm-Starke N. Evidence of diffuse noxious inhibitory controls (DNIC) elicited by cold noxious stimulation in patients with provoked vestibulodynia. *Pain* 2007;130;31-39.
- III. **Johannesson U**, Blomgren B, Hilliges M, Rylander E, Bohm-Starke N. The vulval vestibular mucosa-morphological effects of oral contraceptives and menstrual cycle. *British Journal of Dermatology* 2007; in press.
- IV. **Johannesson U**, Sahlin L, Masironi B, Rylander E, Bohm-Starke N. Steroid receptor expression in the vulvar vestibular mucosa-effects of oral contraceptives and menstrual cycle. *Contraception* Oct 2007; in press.
- V. **Johannesson U**, Sahlin L, Masironi B, Blomgren B, Hilliges M, Rylander E, Bohm-Starke N. Steroid receptor expression and morphology in provoked vestibulodynia. *American Journal of Obstetrics and Gynecology* 2007; in press.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
BLS	Basal layer to surface
BMZ	Basal membrane zone
CGRP	Calcitonin gene-related peptide
COC	Combined oral contraceptives
DNIC	Diffuse noxious inhibitory controls
DPD	Dermal papilla to dermal papilla
DPS	Dermal papilla to surface
DPW	Dermal papilla width
EE	17 α -ethinyl estradiol
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
HADS	Hospital Anxiety and Depression Scale
Ki67	Proliferation factor Ki67
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
NMDA	N-methyl d-aspartate
NS	Nociceptor specific
ns	Non significant
OC	Oral contraceptives
PAG	Periaqueductal grey
PCR	Polymerase chain reaction
PPT	Pressure pain thresholds
PR	Progesterone receptor
QST	Quantitative sensory testing
RCT	Randomized controlled trial
RVM	Rostroventral medulla
SHBG	Sexual hormone binding globuline
SF-36	Short form 36
STT	Spinothalamic tract
TMD	Temporomandibular disorder
VAS	Visual analog scale
WDR	Wide dynamic range

1 INTRODUCTION

“How a few precious facts obscurely come to in the laboratory may resonate into the lives of men everywhere, bring order to disorder, hope to the hopeless, life to the dying. That this is the magic and mystery of our time is sometimes grasped and often missed, but to expound it is inevitable”

Gregory Pincus 1957, after introduction of Enovid- the first oral contraceptive pill.

The etiology of provoked vestibulodynia (former vulvar vestibulitis syndrome), characterized by prolonged severe pain on attempt to vaginal entry, is considered multifactorial. Oral contraceptives are one of the suggested risk factors. An incidence of superficial dyspareunia of 13-16% in young healthy women has been reported. In this period of life relations are committed and the initial need of an effective contraceptive method is generally replaced by a desire for conception. The present studies were initiated due to the clinical observation that many young women seeking help for superficial dyspareunia, did improve simply by omitting their combined oral contraceptives (COC). They displayed an erythematous vulvar vestibular mucosa and in contrast to women with long lasting provoked vestibulodynia, their symptoms disappeared after interrupting the use of the pill. A survey of the literature reveals very few studies concerning the effect of COC on the mucosal lining of the lower genital tract. The results of this thesis are a humble contribution to a puzzle left to complete.

1.1 THE VULVAR VESTIBULE

1.1.1 Anatomy and embryology

The term “vulva” is derived from the Latin word for “covering” (Lewis 2002). The vulva is composed of the anterior mons pubis, the clitoris, the urethral orifice, the labia major and minor, the hymen, the vaginal orifice, Skene’s and Bartholin’s glands and the vestibule. The vestibule is limited anteriorly by the frenulum of clitoris and posterior by the perineum. Laterally the vestibulum extends from the hymen to the inner aspect of labia minora where the inner part of non keratinized squamous epithelium meets the outer part of keratinized epithelium by Hart’s line (Fig. 1).

In the fifth gestational week the anterior urogenital folds form the genital tubercle (Nauth and Haas 1985) which enlarges and forms the clitoris. On either side of the tubercle the labia majora are developed. The urogenital sinus opens up to the urethra, vagina and vestibular glands. The vulvar vestibule is derived from embryonic endoderm whereas the skin-bearing parts are from ectodermic origin.

1.1.2 Histology

The non keratinized vestibular epithelium consists of 20-25 cell layers and the structure resembles that of the vagina and buccal mucosa (Fig. 2) (Woodruff and Friedrich 1985; Sargeant et al. 1996; Thompson et al. 2001; Farage and Maibach 2004). In the stratum superficiale large, flattened cells containing glycogen and frequently pycnotic nuclei are found. The inner cell layers are loosely packed and contain Langerhans cells able to

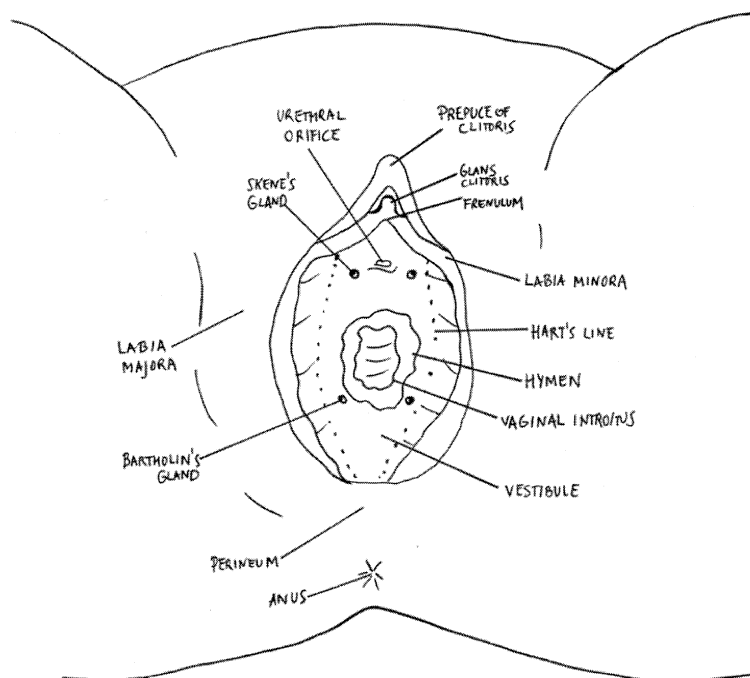


Fig.1. The vulva.

present antigens to circulating T cells. Other immune cells such as lymphocytes are also found intraepithelially and perivascularly and these remain stable throughout the menstrual cycle (Patton et al. 2000). The basal membrane zone (BMZ) is a dynamic junction between the epithelium and the underlying connective tissue. By interlocking the downward projection of the epithelium with the dermal papillae the basement membrane is wrinkled and this profile is specific for different body regions. The vestibular and buccal mucosae have many features in common (Briggaman 1982; Heilman 1987) where an increased permeability to external penetrants is one. This could be due to an absence of a stratum corneum and a lipid barrier with lower resistance to molecular diffusion (Farage and Maibach 2004).

The underlying connective tissue features collagen fibers and capillaries (Lundqvist et al. 1997) whereas the arterioles and venules are found below the lamina propria. The arterial blood flow is derived from the internal iliac and femoral arteries and the venous drainage also reaches these correspondents.

1.1.3 Innervation

Both somatic and autonomic innervation from the sacral nerve roots S2-S4 are present in the vulvar vestibulum where the pudendal nerve carries somatic motor efferents and sensory afferents. The autonomic nerve fibers from the inferior hypogastric plexus and caudal sympathetic ganglia also go through the pudendal nerve (Wesselmann et al. 1997).

The nerve fibers penetrate at the top and along the side of the dermal papillae and intraepithelial free nerve endings have been demonstrated in healthy women (Hilliges

et al. 1995). The intraepithelial free nerve endings in the vestibular mucosa are positive for calcitonin gene-related peptide (CGRP) whereas nerve fibers in the connective tissue express CGRP, substance P, vasoactive intestinal peptide, galanin and neuropeptide Y. The nerve fibers appeared closely to capillaries (Bohm-Starke et al. 1998).

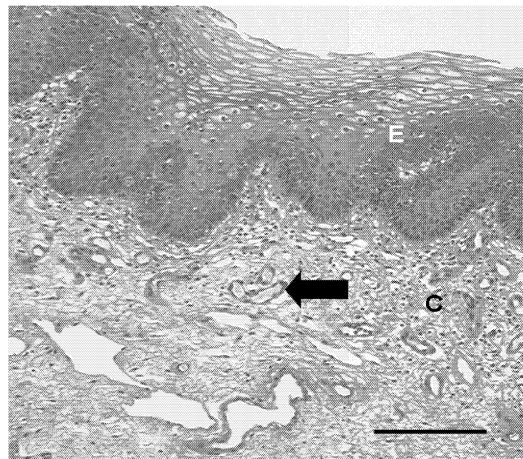


Fig. 2. The vulvar vestibular mucosa with E-epithelium, C- connective tissue. The arrow shows a blood vessel with surrounding endothelial cells.

1.1.4 The menstrual cycle and steroid hormones

The first menstrual cycle (i.e. menarche) usually starts between 9 and 17 years of age and the average length of the cycle is 28 days, however the inter-individual length is 21-35 days. The human menstrual cycle is regulated by an interaction between gonadotropin-releasing hormone (GnRH) from the hypothalamus, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland and the ovarian sex steroid hormones estradiol and progesterone (Speroff et al. 2005a). Figure 3 displays the reproductive cycle with positive and negative feedback loops. The menstrual cycle is divided into two phases. The follicular phase (days 1-14) is characterized by a rising serum 17β -estradiol and low progesterone levels until ovulation, while during the subsequent luteal phase (days 14-28) both serum progesterone and 17β -estradiol will peak (Fig. 4).

By pulsatile secretions of GnRH, FSH from the pituitary gland reaches the ovaries and during each cycle the follicles start to produce estradiol from the granulosa cells. The follicle with the highest number of FSH receptors and granulosa cells becomes dominant and chosen for ovulation. The theca cells produce androgens from cholesterol, serving as a precursor for estradiol production by aromatization in the granulosa cells.

The hormonal fluctuations during the menstrual cycle are reflected in the genital tissues. The endometrium as well as the vaginal mucosa will display various morphological changes (Sjoberg et al. 1988; Nikas et al. 2000). In the endometrium, dilated subepithelial capillaries have been observed in the luteal phase correlating to the

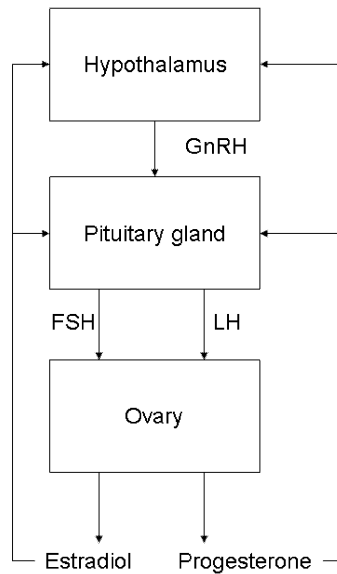


Fig. 3. A simplified model of feedback mechanisms. GnRH releases FSH and LH from the pituitary gland by feedback from estradiol and progesterone. FSH stimulates the ovary to produce estradiol and LH initiates ovulation. There is a negative feedback on FSH at low estradiol concentrations and at higher estradiol levels there is a positive feedback on LH. After ovulation, estradiol and progesterone together exert a negative feedback on FSH, LH and GNRH.

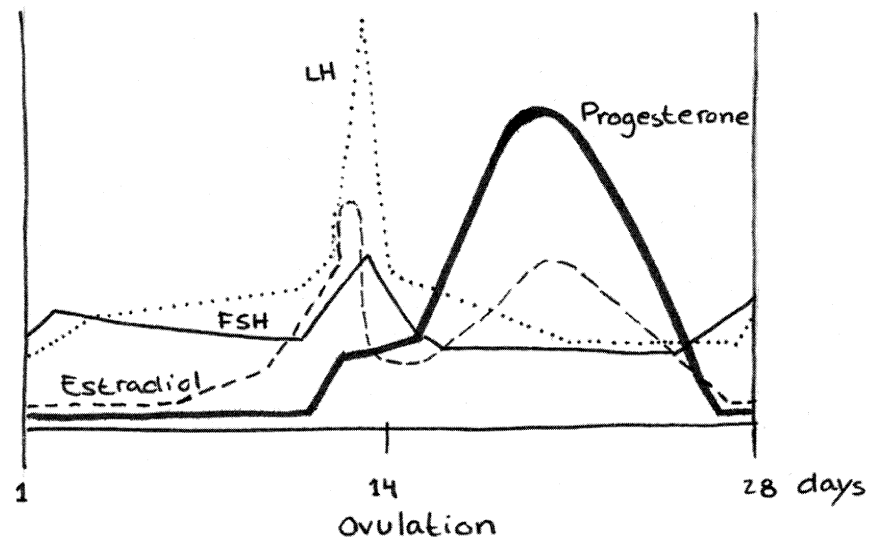


Fig. 4. The menstrual cycle.

1.1.5 Steroid receptors

The steroid receptors are a subgroup of the nuclear receptor superfamily consisting of nuclear receptor proteins with multiple functional domains. They all have a highly conserved DNA-binding domain (C), a hypervariable regulatory domain (A/B), a hinge region (D) important for the movement of the receptor to the nucleus and a hormone binding domain (E) responsible for dimerization and binding to heat shock proteins (Fig. 5). Gene segments are transcribed in the nucleus to messenger ribonucleic acid (mRNA) sequences with a subsequent translation into specific proteins within hours. The non genomic pathway involves interaction with a cell surface receptor and these effects are seen within seconds.

A/B-regulatory domain	C-DNA-binding	D	E-hormone binding	F
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Fig. 5. Schematic model of a steroid receptor. The A/B is the most variable receptor domain. The C-region binds DNA through 2 zinc-fingers. D is the hinge region which plays a role in the movement of the receptor to the nucleus. In region E the hormone binds and dimerization as well as binding to heat-shock protein occurs. The modulation for gene transcription is found in region F.

The effect of the endogenous 17β -estradiol is exerted in a genomic way through estrogen receptor alpha ($ER\alpha$) and estrogen receptor beta ($ER\beta$) and there are also short term non genomic effects primarily in the central nervous system (Wierman 2007). The ERs have two zinc fingers in the C domain and bind to estrogen response elements in the target genes. The ligand-binding domain (E) binds estrogen and estrogenic compounds (Carpenter and Korach 2006). The more recently described $ER\beta$ is partially homologous to the first discovered estrogen receptor $ER\alpha$ (Kuiper et al. 1996). Although $ER\beta$ shares many functional characteristics with $ER\alpha$, the molecular mechanisms regulating the transcriptional activity and the tissue location of $ER\beta$ are different from those of $ER\alpha$. By studying knockout mice the essential roles of these receptors have been clarified in female reproduction (Carpenter and Korach 2006). In most tissues $ER\alpha$ is suggested to have a proliferative effect whereas $ER\beta$ has got an antiproliferative and prodifferentiative effect (Koehler et al. 2005). The $ER\alpha$ is found in the brain, the breast, the cardiovascular system, the liver, bone and in the urogenital tract. The $ER\beta$ has been documented in kidney, brain, bone, heart, lungs, gastrointestinal tract, endothelial cells and in the urogenital tract (Koehler et al. 2005). Progesterone receptor A (PRA) and B (PRB) originate from the same gene. PRA is identical to PRB apart from lacking 164 amino acids from the N-terminal. In most cells, PRB is the positive regulator of progesterone-responsive genes and A inhibits B activity. Repression of the ER transcriptional activity is dependent on PRA. The androgen receptor exists in a full-length A-form and a shorter B-form. The amino acid sequence resembles the progesterone receptor. Progestins and androgens compete for the androgen receptor and for the utilization of the 5α -reductase enzyme. Thereby a progestin may act both as an antiandrogen and an antiestrogen.

Cortisol and other glucocorticoids bind with high affinity to the glucocorticoid receptor (GR). It is expressed in almost every cell in the body. Through GR processes including the development, metabolism, and immune response are controlled (Lu and Cidlowski 2006).

The endometrium, including vascular cells, shows a variation in the expression of the more abundant ER α and the less frequent ER β (Lecce et al. 2001) as well as in PRA and PRB (Mangal et al. 1997) during the menstrual cycle. There is an increase in endometrial ER and PR expression during the proliferative follicular phase and a subsequent decline in the luteal phase (Snijders et al. 1992; Mote et al. 1999; Noe et al. 1999). In the genital tract, the concentration of ER α and PR will gradually decrease from the vagina to vulva whereas the amount of AR is highest in the vulvar skin (Hodgins et al. 1998).

ER and PR mRNA have been extracted from vaginal tissue (Gebhart et al. 2001) and several studies report ER and PR expression in vaginal epithelium (Hodgins et al. 1998; Fu et al 2003). In one study on vaginal epithelium, higher ER concentrations were seen in the follicular phase compared with the luteal phase as measured by enzyme immunoassay (Sjoberg et al. 1989). However, in a more recent study using immunohistochemistry no differences were observed in the vaginal epithelium during the menstrual cycle (Ildgruben et al. 2005). Estradiol up-regulates ER and PR in the uterus whereas progesterone down regulates both receptors during the menstrual cycle (Lessey et al. 1988).

There is a paucity of studies on the steroid receptor expression in the vulvar vestibular mucosa during the menstrual cycle and during COC intake. This is one of the aims of this thesis and will be discussed in detail.

1.2 COMBINED ORAL CONTRACEPTIVES

1.2.1 Ethinyl estradiol and progestins

In 1960 the first combined oral contraceptive (Enovid) was introduced in the U.S. and consisted of the estrogen mestranol (150 μ g) combined with high doses of the progestin norethynodrel (Carr 1997). From then on a multitude of research has expanded the field mainly by reducing the content of estrogen and adding various progestins. By adding an ethinyl group to the parent estrogen 17 β -estradiol, an oral estrogen with prolonged half-life and increased biologic activity is created. The estrogen component has been modified, primarily due to the increased risk of venous thromboembolic events. Combined oral contraceptives contain between 15-50 μ g 17 α -ethinyl estradiol (EE) (Goldzieher et al. 1980). The low-estrogen COC are currently most prescribed and contain less than 35 μ g of EE.

The existing progestins can be divided into three chemical families: derivatives of progesterone; of 19-nortestosterone and gonane; and the newest from spironolactone (Benagiano et al. 2004). These three families are further divided into generations, based upon the time of market introduction. First generation progestins are all derived from testosterone. The gonane progestins can be divided into two categories; the second

generation levonorgestrel and norgestrel and the third generation desogestrel, gestodene and norgestimate (Maitra et al. 2004). The formulations can be of either fixed-dose or phasic combination.

1.2.2 Mechanisms and tissue effects

1.2.2.1 Central effects

COC reduces the secretion of FSH and LH by negative feedback to the hypothalamus and the pituitary gland, thus inhibiting ovulation. EE binds strongly to ER α but in a much lesser extent to ER β (Barkhem et al. 1998; Escande et al. 2006). The progestins bind to the PR, the AR and to the GR with various affinities (Fuhrmann et al. 1995; Garcia-Becerra et al. 2004). The efficacy of COC is very high with a Pearl Index between 0.2 and 3 and they are currently made from synthetic steroids. Some progestins, e.g. norethindrone, can convert to ethinyl estradiol in the body and weakly bind to the estrogen receptor α (Stanczyk and Roy 1990; Kuhl 2005; Garcia-Becerra et al. 2006); however this seems not to be clinically relevant.

The progestin component in synergism with EE reduces the release of gonadotropins at the hypothalamic level by decreasing the number of LH pulses (Speroff et al. 2005b) (Fig.4).

1.2.2.2 Peripheral effects

The genital organs are affected primarily by the progestins in a dose-dependent manner compared with EE (Speroff et al. 2005b). Estrogen alone causes proliferation and thickening of the endometrium. However, a combination of EE and progestins causes decidual reactions and after a period of influence an atrophied mucosa which will inhibit implantation (Rabe et al. 1997; Ludicke et al. 2001; Grow and Iromloo 2006). The expression of steroid receptors in the endometrium during the use of combined oral contraceptives has been insufficiently investigated.

Progestins will decrease the amount, ferning, viscosity and spinnbarkeit of cervical mucus (Hull and Moghissi 1986). The mucus resembles that seen during the end of pregnancy (Chretien et al. 1991).

In a study on ovariectomized macaques a significant increase in vaginal keratinization and vaginal secretion was seen after treatment with ethinyl estradiol only (Sikoski et al. 2007). The percentage of vaginal superficial cells, also known as the karyopycnotic index, changes during the use of COC (Kauraniemi and Hirvonen 1974). A low karyopycnotic index with distended superficial cells in the vaginal epithelium was observed in a small study of COC users (Katira and Dayal 1987) but the contrary was found in a cytomorphometric analysis (Chretien et al. 1998). Another conflicting finding is that depot-medroxyprogesterone acetate has been shown to cause a slight thinning (Miller et al. 2000), as well as a thickening of the vaginal epithelium (Ildgruben et al. 2003). In COC users both an unchanged (Eschenbach et al. 2000) vaginal epithelium as well as a thicker one has been observed (Ildgruben et al. 2003).

1.3 PERIPHERAL AND CENTRAL PAIN MECHANISMS

Pain is defined as a reaction of the somatosensory system to noxious stimuli, serving to protect the individual from tissue damage. The pain system can be altered by injury,

disease and genetic factors, causing a disturbed functioning. Fear and anxiety will modify its quality. Pain may become persistent and debilitating with reduced pain thresholds and increased response to stimulation. Long-lasting pain promotes a destructive stress response affecting the physical and mental health of the person (Chapman et al. 1999). Pain intensity can be modulated in the periphery as well as in the central nervous system (DeLeo 2006).

1.3.1 Nociceptors and nerve fibers

Nociceptors are free nerve endings present in most tissues of the human body. They are responding to noxious mechanical, thermal and chemical stimulation. During inflammation, release of endogenous neuropeptides can sensitize and decrease pain thresholds in C-nociceptors (Lee et al. 2005).

Pain is transmitted through the thin myelinated A δ -fibers and the unmyelinated C-fibers. The A δ -fibers rapidly transmit discriminative information which can lead to a reflex flexion withdrawal (Julius et al. 2001) and this will result in sharp and localised pain. Thereafter the C-fibers with less conduction velocity will give rise to a secondary aching pain.

1.3.2 Dorsal horn

The primary afferent neurons are mainly terminating in the lamina I, II and V of the dorsal horn. A modulation of information can occur before further transmission to the brain. The nociceptor-specific (NS) neurons convey precise information about peripheral location, while wide-dynamic range neurons (WDR) receive input from sensory receptors over a large area (Fig. 6). The WDR can encode the intensity of noxious stimuli but have less capacity to distinguishing noxious from innocuous stimuli. They are under considerable control by supraspinal centres. At this level, synaptic connections are made to efferents of skeletal muscles and sympathetic nerve fibers (Hökfelt et al. 1975; Le Bars 2002).

1.3.3 Supraspinal centres

The ascending spinothalamic tract (STT) projects to parts in the brain such as the lateral and medial nuclei of the thalamus. Axons that terminate in the thalamus can project to the frontal or somatosensory cortex. The anterior cingulate cortex integrates information about pain perception. The spinoreticular tract ascends from the dorsal horn to the reticular formation in the brain stem (Cross 1994). The reticular formation is partly responsible for the autonomic response of pain; sweating, changes in pulse, blood pressure and respiratory rate (Almeida et al. 2004).

1.3.4 Pain modulation

Pain can be decreased or increased by different mechanisms. There are two crucial receptors engaged in this; the inhibitory GABA-receptor and the excitatory NMDA-receptor (Dickenson et al. 1997). Descending pain modulation can be either inhibitory or facilitatory (Zhuo and Gebhart 1992; Gebhart 2004). Recent evidence support that the facilitation mechanisms can contribute to hyperalgesia and chronic pain syndromes. Figure 7 displays an overview of peripheral and central components of antinociception (DeLeo 2006). Hyperalgesia is defined as an altered pain perception where a stimulus

which would normally induce a light pain causes significant pain. Allodynia is a painful response to a stimulus that does not normally elicit pain (DeLeo 2006).

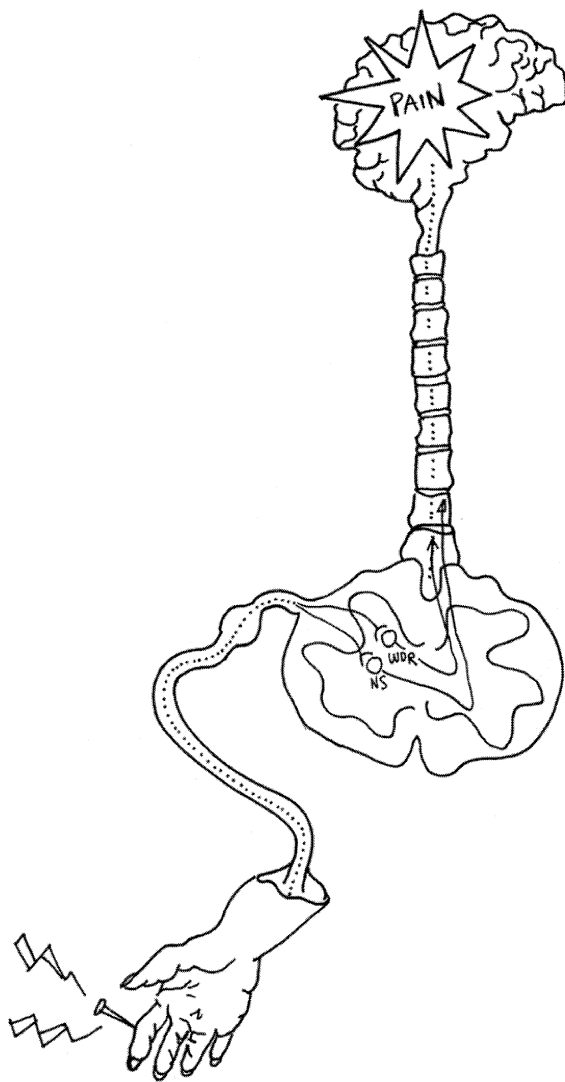


Fig. 6. The pain pathway through nociceptive-specific (NS) and wide dynamic range (WDR) neurons in the dorsal horn.

1.3.4.1 Peripheral modulation

Tissue damage results in a drop of pH and release of prostaglandins, substance P, bradykinin, histamine, potassium and leukotrienes which all are capable of sensitizing the small non-myelinated C fibers. Threshold activity of the C fibers may be up-regulated peripherally by serotonin, prostaglandins, thromboxane, and leukotrienes in the damaged tissue. This is referred to as peripheral sensitization in contrast to central sensitization which occurs at the dorsal horn in the spinal cord and in the brain (Fig 8). Substance P may also be released peripherally with resultant increase in peripheral vasodilatation and further sensitization of the nociceptor. Non steroidal anti-inflammatory drugs (e.g. NSAID) will decrease the production of prostaglandins and will consequently modulate pain (DeLeo 2006).

1.3.4.2 Spinal modulation

The peripheral input to the dorsal horn can modify the flow of impulses to higher processing centres, a procedure known as “the gate control theory” (Dickenson 2002). Incoming signals in the A β fibers of a peripheral nerve can alter the sensitivity of the post-synaptic cells to painful stimuli arriving in C and A δ fibers (Melzack and Wall 1965).

1.3.4.3 Supraspinal modulation

One supraspinal pain modulatory mechanism is due to diffuse noxious inhibitory controls (DNIC) which can be summarized as “pain inhibits pain” (Le Bars et al. 1979). By application of a second painful stimulus (cold, heat, electricity etc), distant from the neuron under study, the first pain (pressure pain, hypertonic saline injections etc) is reduced (Le Bars 2002). DNIC is supraspinally mediated through an inhibition of wide dynamic range and nociceptive specific neurons in the dorsal horn (Hu 1990; Le Bars 2002).

The descending inhibitory pathways emerge from the rostroventral medulla including the subnucleus reticularis dorsalis and nucleus raphe magnus. They are mainly modulated by noradrenaline and serotonin, by which the release of substance P is inhibited in the substantia gelatinosa (lamina II) of the dorsal horn (Pertovaara 2006). This activates enkephalinergic interneurons that inhibit nociception at the spinal level and the effect can consequently be inhibited by naloxone (Lundeberg 1995). In a recently published study it was observed that DNIC-like pain inhibition was unaffected by lorazepam which bind to the GABA_A-receptor (Kunz et al. 2006). DNIC has been suggested not only as a mere pain relief but also as a phenomenon associated with the production of withdrawal reflexes (Price and McHaffie 1988).

1.3.4.4 Cortical modulation

The cortex might reduce pain by activating the descending pain modulatory systems located in the brainstem. It has also been suggested that different cortical areas, probably through GABA, can change the affective aspect in order to decrease the aversive component of pain (Ohara et al. 2005; Price et al. 2006). Another theory on

pain modulation is the neuromatrix theory which proposes that pain is genetically determined and modified by sensory input (Melzack 2004).

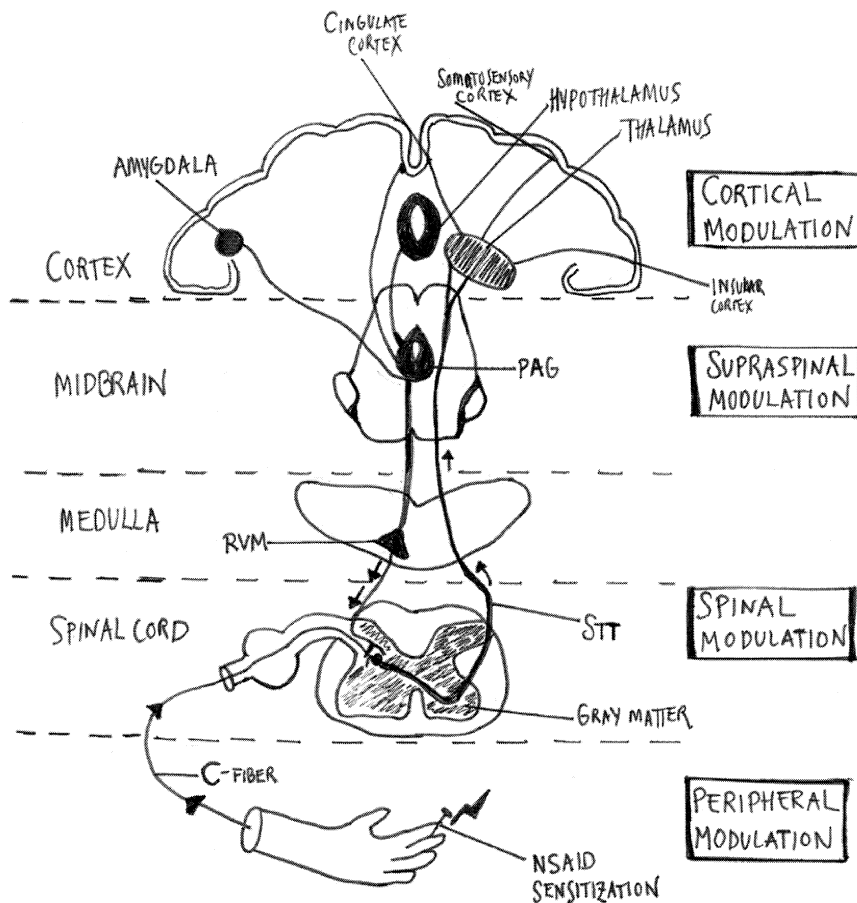


Fig. 7. Endogenous pain modulation. PAG-periaqueductal gray, RVM-rostromedullary. Ascending information in the STT-spinothalamic tract.

1.3.4.5 Clinical aspects

In subjects with temporomandibular disorder (TMD), characterized by regional chronic pain, the endogenous pain inhibitory systems are impaired (Maixner et al. 1995; Kashima et al. 1999; Ge et al. 2004). A deficient DNIC mechanism has also been found in patients with chronic tension type headache (Pielsticker et al. 2005). Similar findings are reported in more generalized pain conditions such as fibromyalgia (Kosek and

Hansson 1997; Lautenbacher and Rollman 1997). A lack of DNIC response has been suggested as a risk factor for the development of longstanding pain (Leffler et al. 2002a; Edwards et al. 2003b) supporting the importance of endogenous pain modulation in clinical pain.

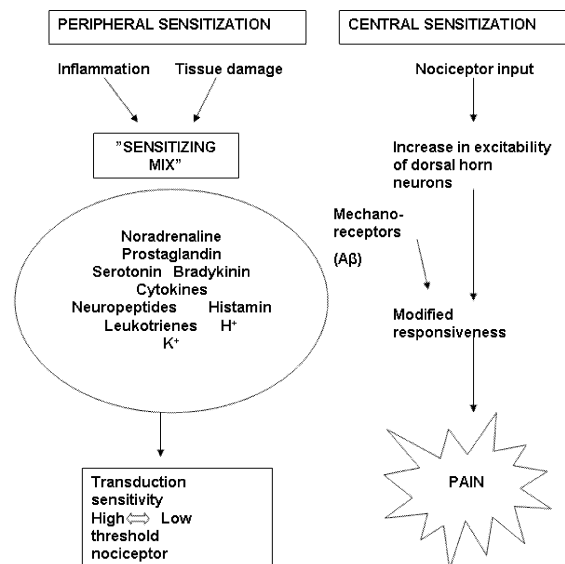


Fig. 8. Peripheral and central sensitization.

1.3.4.6 Pain and hormones

Many chronic pain conditions are more prevalent in women than in men, which may signify an influence of steroid hormones. In a study on rats, a sexual dimorphism was found in the opioid-mediated pain inhibitory mechanism (Gaumond et al. 2007). Estrogen and inflammation can increase the excitability of afferent neurons in rats (Flake et al. 2005). In humans, higher pain thresholds and pain tolerance have been observed more often during the follicular phase, with high circulating estrogen levels as compared to the luteal phase when also progesterone is secreted (Riley et al. 1998). However, it is currently questioned if pain perception varies across the menstrual cycle (Sherman and LeResche 2006).

Studies on oral contraceptives (OC) as a contributing factor to clinical and experimental pain are discordant. In one study on myofascial pain the pain levels were more constant in users compared with nonusers (Dao et al. 1998) and in another study the use of OC was associated with a 20% increased risk of TMD (LeResche et al. 1997).

Isselee et al. 2001 showed that pressure pain thresholds of masticatory muscles were significantly lower in the perimenstrual phase in both COC-users and nonusers (Isselee et al. 2001). Moreover, it has also been observed that menstruating women had higher pain thresholds compared with OC users and men (Kowalczyk et al. 2006). Endogenous pain modulation measured as DNIC has not been investigated in provoked vestibulodynia or correlated to COC use, although OC use has been suggested as a possible etiological factor for provoked vestibulodynia with an increased risk of 9.3 in young users (Bouchard et al. 2002).

1.4 PROVOKED VESTIBULODYNIA

1.4.1 Definition

Long-lasting superficial dyspareunia is a common gynecological problem in young women where the majority is diagnosed with provoked vestibulodynia (former vulvar vestibulitis syndrome) (Meana et al. 1997; Danielsson et al. 2003; Harlow and Stewart 2003; Haefner et al. 2005). It is an increasing clinical problem affecting the daily life and psychosexual well being of sufferers (Danielsson et al. 2001).

Provoked vestibulodynia is defined as a localized pain syndrome in the area around the vaginal opening causing severe pain at any attempt of vaginal entry (Friedrich 1987; Moyal-Barracco and Lynch 2004) and erythema of various degrees may also be present. In contrast to many chronic pain disorders characterized by spontaneous ongoing pain, the pain experienced in vestibulodynia is primarily provoked by sexual activity, tampon use or in some cases tight clothing.

Some of the affected women show signs of anxiety and depression and during gynecological examination increased tension of the pelvic floor (Abramov et al. 1994; Har-Toov et al. 2001).

Primary provoked vestibulodynia refers to a situation with pain since the first attempt of vaginal entry (tampon or intercourse). In this category of women a history of psychological factors are more common (Harlow and Stewart 2005). In the majority of women diagnosed with provoked vestibulodynia the secondary form prevails and pain develops after a period of pain-free intercourse.

1.4.2 Etiology

The etiology of provoked vestibulodynia remains an enigma, and like other pain disorders there might be several triggering factors. Although the research field has expanded, the current theory is based on a multifactorial etiology (Zolnoun et al. 2006). Several researchers have analyzed the connection between recurrent candida infections and provoked vestibulodynia (Bazin et al. 1994; Nyirjesy 2001; Berglund et al. 2002). The majority of the women diagnosed at the vulvar clinic at Danderyd Hospital also reported recurrent episodes of candida vulvovaginitis (Bohm-Starke and Rylander 2000). Other infectious agents such as human papilloma virus (HPV) have been studied without finding a possible association (Green et al. 2001).

Previous treatments in this area have also been suggested as a pain triggering factor. Irritants used in HPV treatment and contact allergy to various products may cause

microlesions to the mucosa and initiate a pain condition in some women (Farage and Maibach 2004).

Psychosexual factors may contribute to the development of vestibulodynia (Sackett et al. 2001). However, in numerous studies it was observed that a history of sexual abuse is not more prevalent in women with provoked vestibulodynia than in healthy control subjects (Danielsson et al. 2000; Granot and Lavee 2005). Nevertheless, in a Boston study significantly more women with vulvar pain reported histories of physical abuse, but only 50% of the interviewed underwent a clinical examination (Harlow and Stewart 2003). In the literature it is argued whether the psychological dysfunction is a consequence of longstanding pain (Nunns and Mandal 1997) or if it is primarily a somatization disorder (Mascherpa et al. 2007).

Genetic variables such as the presence of interleukin-1B gene polymorphism, resulting in a prolonged pro-inflammatory immune response (Witkin et al. 2002; Gerber et al. 2003) and also less potent antiinflammatory counterparts such as interleukin-1 (Jeremias et al. 2000; Foster et al. 2004) have been observed in women with vestibulodynia. There have also been reports on an altered distribution of mannose-binding lectin, which is active in the innate immune defence against micro organisms (Babula et al. 2004).

The use of oral contraceptives at an early age and for a long duration has been identified as a risk factor for development of provoked vestibulodynia (Berglund et al. 2002; Bouchard et al. 2002). Lower levels of ethinyl estradiol in some COC have been suggested to contribute to vaginal dryness and dyspareunia (Caruso et al. 2004).

Hypertension of the pelvic floor may be a secondary protective reflex to a pathologic process in the vestibular mucosa (Graziottin and Brotto 2004; Reissing et al. 2004). It might also be due to sensitization of pain fibers which will cause contraction of the underlying muscle (Graven-Nielsen and Arendt-Nielsen 2002).

Peripheral sensitization of the vestibular sensory nerves (Bohm-Starke et al. 2001c) as well as lower systemic pain thresholds (Granot et al. 2002; Pukall et al. 2002; Giesecke et al. 2004; Granot et al. 2004; Foster et al. 2005) and an altered central sensory processing (Pukall et al. 2005) have been reported. These findings may be compatible with a concomitant central sensitization leading to an enhanced pain perception in vestibulodynia patients. The endogenous pain modulation has not been investigated in these patients.

1.4.3 Histopathology

Histopathology of vestibular biopsies from women with provoked vestibulodynia has shown unspecific inflammation (Pyka et al. 1988; Bornstein et al. 2004). However, similar findings are also present in the vulvar tissue of healthy women (Lundqvist et al. 1997). In a recently published study it was shown that strains of fibroblasts from women with provoked vestibulodynia do express inflammatory cytokines in a higher extent compared with those of healthy women. This observation was interpreted as a sign of inflammation (Foster et al. 2007). Contradictory, lower levels of inflammatory markers were found in vestibular biopsies in women with vestibulodynia than in healthy controls (Eva et al. 2007). Routine histopathological evaluation is currently not used for diagnosis, but an increase in peripheral nerve fiber density in the vestibular mucosa has been described (Bohm-Starke et al. 1998; Westrom and Willen 1998;

Tympanidis et al. 2003). An increase of the pain related vanilloid receptor as well as an increase in blood flow in the vestibule has been found (Bohm-Starke et al. 2001b; Tympanidis et al. 2004). These latter findings do support the theory of neurogenic inflammation in these women and may explain the reported lower pain thresholds (Pukall et al. 2002; Giesecke et al. 2004). Eva et al. found low levels of estrogen receptor alpha in the vestibular mucosa of women with provoked vestibulodynia (Eva et al. 2003). However, no investigators have analyzed the presence and distribution of the other steroid receptors.

1.4.4 Treatment

Currently, treatment is focused on pain management through several modalities.

1.4.4.1 Topical treatments

Topical lidocaine blocks the transmission in C-fibers resulting in desensitization of peripheral nociceptors in the vestibular mucosa. Topical lidocaine in a 2% gel or a 5% ointment overnight or several times daily have been used with promising results (Danielsson et al. 2006; Zolnoun et al. 2006).

Cromolyn cream inhibits mast cell degranulation and release of mediators, but has not been proven more efficient than placebo in a randomized controlled trial for vestibulodynia (Nyirjesy et al. 2001).

Topical estradiol and estriol cream have been tried although there is a lack of published randomized studies and the same concerns topical testosterone and nitroglycerin.

Topical nitroglycerin cream has been tested in a pilot study on vulvodynia patients with headache as a major side effect causing discontinuation (Walsh et al. 2002).

Application of a capsaicin cream caused a partial response but the main side effect (severe burning) resulted in discontinuation (Murina et al. 2004).

1.4.4.2 Injection treatments

Infiltration with dexamethasone and other glucocorticoids are today not considered as an effective treatment even though some success has been reported in small uncontrolled studies (Sonnex 1999; Murina et al. 2001; Segal et al. 2003).

Based on the earlier hypothesis of HPV as an etiological cause, injections of interferon were tried (Bornstein et al. 1993). Due to flu-like symptoms and low success rates this is no longer a treatment option (Marinoff and Turner 1991).

Botulinum toxin injections causing reversible paralysis of the bulbocavernosus muscle have been tried with great success but on a limited number of patients in uncontrolled studies (Romito et al. 2004; Dykstra and Presthus 2006; Yoon et al. 2007).

1.4.4.3 Tricyclic antidepressants and anticonvulsants

The tricyclic antidepressant amitriptylin is a potent pain reducing agent in patients with generalized vulvodynia (Reed et al. 2006) and is the most common treatment for vestibulodynia, despite the lack of randomized controlled trials (RCT) (Haefner et al. 2005).

The anticonvulsant gabapentin has mainly been applied in the treatment of unprovoked vulvodynia (Ben-David B 1999). In a recently published study an 80 % pain reduction was seen in 64% of patients with unprovoked vulvodynia, however this was not a RCT

(Harris 2007). Although there have been reports of anorgasmia in gabapentin users (Grant and Oh 2002), the drug might be tried when amitriptyline has been ineffective (Bates and Timmins 2002).

1.4.4.4 Physical therapy, including EMG-biofeedback and acupuncture

Since the pelvic floor musculature may play a role in the maintenance of provoked vestibulodynia, several studies have sought to evaluate the effectiveness of physical therapy (Bergeron et al. 2002b; Hartmann et al. 2007). By increasing awareness and proprioception the patients can be taught to improve their muscle strength and endurance leading to a decrease of hypertonicity and increase of voluntary relaxation. The fear of vaginal penetration can also be diminished. Manual techniques as well as patient home exercise are applied but the most promising treatment has been EMG biofeedback (Glazer et al. 1995; Hartmann et al. 2007). EMG biofeedback has been reported to decrease pain during intercourse and resting tension as well as restore stability of the pelvic floor muscles after 16 weeks of practice in women with vestibulodynia. The re-establishment of the muscle function with an improved capacity to relax the pelvic floor during sexual activity is thought to reduce the coital pain. A contracted muscle containing lactic acid and various sensitizing substances might theoretically influence the peripheral nociceptors.

In a small Swedish study the quality of life was improved in women with vestibulodynia who received acupuncture treatment (Danielsson et al. 2001), but larger studies are required.

1.4.4.5 Psychosexual counseling

Cognitive behavioural therapy including a combination of pain management and sexual therapy has been proven effective in several studies with equivalent treatment outcome as vestibulectomy (Bergeron et al. 2001).

Other studies on psychological interventions such as hypnotherapy have reported less pain and increased sexual functioning (Kandyba and Binik 2003).

1.4.4.6 Surgery

A surgical procedure called perineoplasty was described in 1981 (Woodruff et al. 1981). This technique, by which the posterior part of the vestibule is removed and replaced by vaginal tissue (partial vestibulectomy), has been modified and there are about 20 studies published regarding surgery (Bornstein et al. 1997; Haefner et al. 2000). There is an ongoing debate concerning when it should be used and many consider this as a last option restricted to those patients where all other treatments have been unsuccessful (Schneider et al. 2001; Traas et al. 2006). However, vestibulectomy is still performed with the success rate of 50-100%, including complete and partial response. In most studies the success rate is related to the degree of persisting introital dyspareunia (Bergeron et al. 2001). Nevertheless, a significant improvement in the quality of sexual life was also observed recently. Remarkably, significantly more women who had a new partner reported a complete cure than those who did not change partner during the follow-up period. Women with primary vestibulodynia will generally not benefit from surgery (Bornstein et al. 1997; Bohm-Starke et al. 2007, in press).

2 AIMS

- To analyze the somatosensory perception in the vestibular mucosa in healthy women with and without combined oral contraceptives.
- To investigate whether women with provoked vestibulodynia display DNIC responses to cold noxious stimuli and if their responses differ from those in healthy women using or not using combined oral contraceptives (COC).
- To evaluate the morphology of the vulvar vestibular mucosa in healthy women during the influence of COC and during the menstrual cycle.
- To analyze the presence of steroid receptors in the vestibular mucosa during usage of COC and during the menstrual cycle in healthy women.
- To investigate whether the morphology of the vulvar vestibular mucosa and its expression of steroid receptors differ in women with provoked vestibulodynia from that of healthy controls.

3 PARTICIPANTS

3.1 ETHICS

The studies were approved by the local Ethics Committ  at Karolinska Institutet and all subjects gave their written and informed consent.

3.2 SUBJECTS

In all, we have studied 34 women with provoked vestibulodynia, 60 healthy women using combined oral contraceptives and 64 healthy women not using combined oral contraceptives.

3.2.1 Women with provoked vestibulodynia

The patients were recruited from the outpatient vulvar clinic at Danderyd Hospital. They had been referred from other gynecologists in the Stockholm area for superficial dyspareunia. All the women fulfilled Friedrich's diagnostic criteria for vulvar vestibulitis syndrome (i.e. provoked vestibulodynia) (Friedrich 1987; Moyal-Barracco and Lynch 2004).

Thirty-four women with provoked vestibulodynia were included in study II and V. Most patients had previously used combined oral contraceptives (COC) but they had omitted these at least 6 months prior to the studies. Inclusion criteria for the patients were: provoked dyspareunia confined to the vaginal opening, pronounced pain during most intercourse attempts, duration of symptoms \geq six months, condoms as the only contraceptive method and age \geq 18 years.

Exclusion criteria for the patients were regular use of analgesics or antidepressants, pregnancy, bleeding disorders, vulvar dermatoses and systemic diseases such as hypertension and diabetes.

To exclude an ongoing vulvovaginal infection, a careful gynaecological examination was made, with wet mount and cultures if needed. Coitus was asked to be avoided 48 hours prior to when the biopsies were taken (study III-V). A socio-medical profile was obtained by a standardized and coded questionnaire.

3.2.2 Healthy women with and without use of COC

Sixty healthy control subjects using COC and 64 not using COC were recruited mainly among university students through local advertisements. Forty five of them participated in more than one study (Table 1). The COC used in all studies contained 20-35 μ g ethinyl estradiol and various progestins and the women had used them for a minimum of 6 months prior to the studies (Table 2). The pills were both monophasic and multiphasic regarding the progestin and ethinyl estradiol content. Inclusion criteria for all control subjects were; healthy and sexually active women, age 18-35 years and condoms as the only contraceptive method. Additional inclusion criteria for the healthy women without COC were a history of regular monthly menstrual cycles. Exclusion criteria for the healthy women were: dyspareunia, regular use of analgesics or antidepressants, pregnancy, bleeding disorders, vulvar dermatoses and systemic diseases such as hypertension and diabetes. A careful gynaecological examination was made, with wet mount and cultures if needed, to exclude an ongoing vulvovaginal

infection. Coitus was asked to be avoided 48 hours prior to when the biopsies were taken (study III-V). A socio-medical profile was obtained by a standardized and coded questionnaire.

Table 1. Participants reported in papers I-V

Study I-V	I	II	III	IV	V
I	COC=20 nonCOC=19	-	-	-	-
II	-	P=20 COC=20 nonCOC=20	-	-	-
III	-	-	COC=20 nonCOC=25	COC=20 nonCOC=25	- nonCOC=25
IV	-	-	COC=20 nonCOC=25	COC=20 nonCOC=25	- nonCOC=25
V	-	-	-	-	P=14 nonCOC=25

3.2.3 Clinical data

Clinical data on patients and healthy women using or not using combined oral contraceptives are presented in Table 3. The patients frequently reported previous use of OC and various bodily pains. Both patients and healthy subjects reported a history of Candida infections.

Table 2. Ethinyl estradiol and progestin content of the combined oral contraceptives (COC) used in study I-IV.

COC brand	Ethinylestradiol µg	Progestin µg	Number of subjects
Trionetta®	30-40-30	L-50-75-125	10
Trinovum®	35	N-500-750-1000	8
Mercilon®	20	D-150	10
Cilest®	35	NGM-250	3
Follimin®	30	L-150	7
Restovar®	37.5	LY-750	2
Yasmin®	30	DR-3000	3
Desolett®	30	D-150	7
Orthonett- Novum®	35	N-500	3
Synfase®	35	N-500-1000-500	1
Trinordiol®	30-40-30	L-50-75-125	1
Diane®	35	C-2000	1

Progestin abbreviations: L= levonorgestrel, N= noretisterone, D= desogestrel, NGM= norgestimate, LY= lynestrenol, DR= drospirenone, C= cyproteronacetate.

The brand names are registered in the annual pharmacy declaration in Sweden, FASS® and the pharmaceutical companies are Schering Nordiska (Trionetta®, Yasmin® and Diane®), Janssen-Cilag (Trinovum®, Orthonett-Novum® and Cilest®), Organon (Mercilon®, Desolett® and Restovar®), Pfizer (Synfase®), and Wyeth (Follimin® and Trinordiol®).

Table 3. Clinical data on subjects.

Clinical variables	Patients (n=34) years and %	Non COC (n=64) years and %	COC (n=60) years and %
Age	24.1(19-33)	24.9 (18-33)	22.7 (18-34)
Previous OC	91	84	100
Present OC	0	0	100
Debut age OC	18.6 (16-23)	19 (16-24)	17.3 (16-25)
Years of OC	4.6 (0.5-10)	3.6 (0.5-11)	5.3 (0,5-15)
Dyspareunia	5.2 (1-18)	0	0
Headache	56	31	30
Muscle ache	35	16	20
Back pain	45	15	0
Dysmenorrhea	65	38	38
Candida infection	68	40	45

4 METHODS

The aim was to study the impact of combined oral contraceptives (COC) on the vulvar vestibular mucosa and pain mechanisms in healthy women and in women with provoked vestibulodynia (former vulvar vestibulitis syndrome). Sensory function of the peripheral vestibular nerves, endogenous pain modulation, morphology and steroid receptor expression in the vulvar vestibular mucosa were studied. The methods are listed in Table 4.

Table 4. Methods used in studies I-V

Methods	I	II	III	IV	V
Quantitative sensory tests	X				
Pressure pain		X			
Cold pressor test		X			
Biopsies			X	X	X
Immunohistochemistry				X	X
Epithelial measurement			X		X
Histopathology			X	X	X
Questionnaire	X	X	X	X	X
HADS		X			
SF-36		X			

4.1 QUANTITATIVE SENSORY TESTING (I)

Quantitative sensory testing (QST) is often used in neurophysiologic units in order to determine abnormalities in sensory and pain perception (Wahren and Torebjork 1992). Thermal and mechanical stimuli are used to determine the perceptual and pain thresholds for each type of stimuli (Dyck et al. 2000).

QST was performed on the vulvar vestibular mucosa in healthy women with and without combined oral contraceptives on days 7-11 of the menstrual cycle to standardize for any difference in pain perception (Riley et al. 1998). Two areas of the vestibular mucosa were tested on the right side by an examiner who was blinded to whether the subjects were using COC or not (Fig. 9). Area A, in the anterior vestibule is generally not as painful as area B in the posterior vestibule proximal to the Bartholin's gland (Eva et al. 1999, Bohm-Starke et al. 2001c). Von Frey filaments were used to determine pain thresholds for punctate mechanical stimulation (Fig. 10). The monofilaments represented an applied force from 1.2 to 575 mN. They were applied perpendicularly 2-3 times each on the mucosal surface. The subjects reported verbally the first sensation of pain. The weakest von Frey filament eliciting pain on 2 of 3 applications was defined as the pain threshold to be used for statistical analysis.

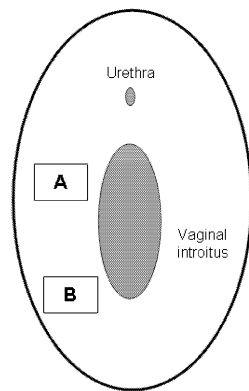


Fig. 9. The vestibular areas investigated by QST.

The thermotest included detection thresholds for warmth, cold and pain threshold for heat and was performed by using the Marstock method (Somedic AB, Stockholm, Sweden) (Fig. 10). A 7x15 mm area of the vestibular mucosa was heated or cooled using a thermal stimulator with semiconductor junctions operating on the Peltier principle (Fruhstorfer et al. 1976). After 3-5 stimuli a mean value of the thresholds was obtained. Starting from an adapting temperature of 31°C, the rate of temperature change was 0.5°C/sec for thresholds for warmth and cold and 1°C/sec for heat pain threshold. The range tested was 6-50°C. A training session was carried out in all subjects in non-affected skin in the thenar eminence or on the thigh before the vestibular mucosa was investigated.

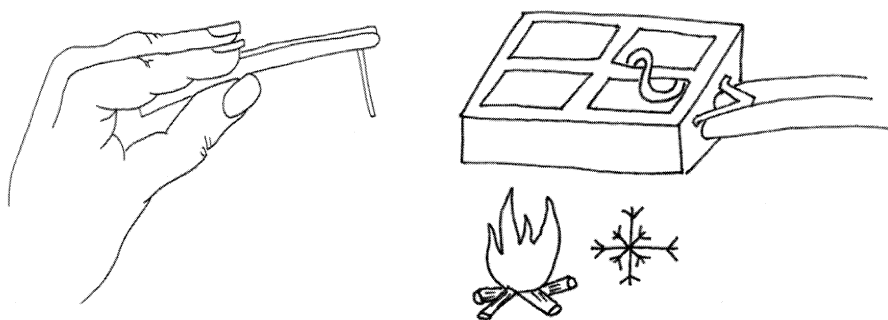


Fig. 10. To the left the hand-held von Frey filament used for punctate mechanical stimulation and to the right the thermal stimulator with an area of 7x15 mm.

The contact plate was lightly applied to the mucosal surface in areas A and B. In the primary session the subjects were instructed to signal the first sensation of warmth and cold, changing the direction of thermal stimulation by pressing a button. Secondly, the pain thresholds for heat were determined. The subjects were instructed to press the button when the first sensation of pain occurred. In all cases thresholds for thermal perception and heat pain were first determined in A and then in B. Cold thresholds were then determined in A and finally in B, in order to avoid paradoxical heat sensations.

4.2 PRESSURE PAIN THRESHOLD ASSESSMENT (II)

The test was performed on one occasion in the follicular phase, days 7-11 after onset of the last bleeding, to standardize for any differences in pain perception related to the menstrual cycle.

Two investigators blinded to whether the subject was a patient or not, conducted all assessments after having tested the procedure on a five healthy subjects not included in this study. During the testing phase there were no observed differences in pain threshold measurements between the two investigators. All subjects were given a careful explanation of the procedure before the testing started. The subjects were sitting upright in a chair in a room without distracting visual stimuli (Fig.11). A metal pressure algometer (Somedic Sales AB Hörby, Sweden) with a disk shaped rubber top of 1 cm² was used. Pressure pain thresholds (PPT) were measured by applying the device perpendicularly to the skin surface. The induced pressure was between 0-2000 kPa, increased by a velocity of approximately 50-75kPa/s and displayed digitally. The women were instructed to press a button to interrupt the procedure when pressure discomfort turned into a painful sensation. At this moment, defined as the PPT, pain intensity was scored on a visual analogue scale (VAS) 0-100, with 0 representing no pain and 100 the most intense pain imaginable. Two different body areas were tested on the opposite side of the subjects' reported dominant hand. The leg was first tested on the anterior tibial muscle, approximately 5 cm below and 3 cm lateral to the tibial tuberosity. Subsequently the pain threshold of the upper arm was tested on the deltoid muscle, 3 cm proximal to the tendon insertion on the humerus.

4.2.1 Induction of DNIC response

After the initial pressure pain stimulation on the leg and arm, the dominant arm was fully immersed into ice water with a mean temperature of 3.3° C (range 2-5) during one minute (Fig.11). The ice water was kept in a thermo box to keep a constant temperature throughout the session. The cold-induced pain intensity was scored after 45 seconds using VAS. Immediately thereafter the assessments of PPT and pain intensity of the contra lateral leg were repeated according to the protocol but with the dominant arm remaining in ice water. The arm was thereafter removed from the ice water and the assessment of PPT of the contra lateral arm was repeated including VAS.

A DNIC response was defined as an increase in PPT within each subject directly after cold noxious stimulation.

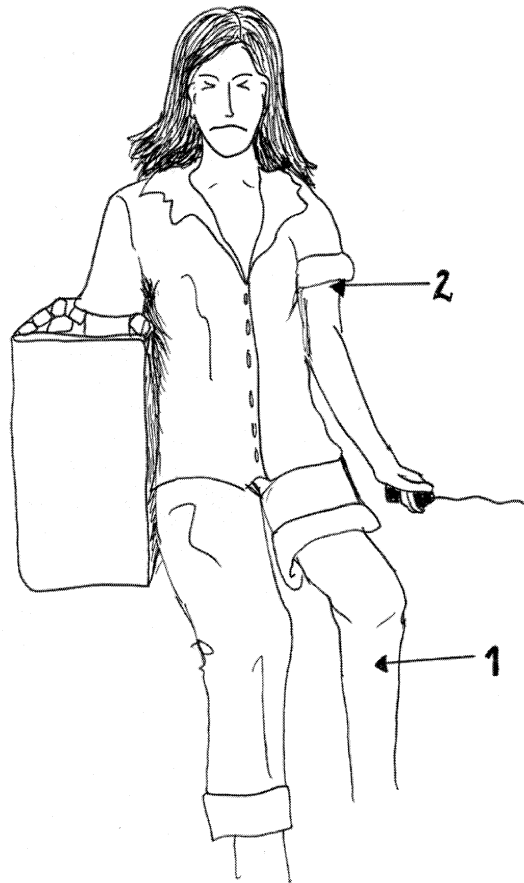


Fig. 11. The DNIC procedure. 1-the leg area tested first for pressure pain threshold, 2- the arm area tested subsequently.

4.3 HADS AND SF-36 (II)

The Hospital Anxiety and Depression Scale (HADS) was used to detect anxiety disorders and depression. HADS has been found to be a validated screening instrument for anxiety and depression in both somatic and primary care patients as well as in the general population (Herrmann 1997; Bjelland et al. 2002). It is a questionnaire composed of 7 statements relating to generalized anxiety and 7 statements for depression. Each statement is ranked from 0-3, with 0 representing no symptoms and 3 considerable symptoms. The maximum score for anxiety and depression is 21 with a score of 8-10 indicating mood affection and a score of 11 and above suggesting presence of mood disorder (Snaith 2003).

Short-form-36 (SF 36) is a health survey measuring functional impairment and well-being assessing nine domains. The items Physical functioning, Role limitations due to

4.4 BIOPSIES (III, IV,V)

4.4.1 Sampling

Serum samples for estradiol and progesterone were collected in order to determine the current phase of the menstrual cycle of the participants. The samples were analyzed by an immunoassay technique (DxI, Beckman-Coulter, Bromma, Sweden). Biopsies from the vulvar vestibule were taken days 7-11 since the first day of the menstrual period in all participants. After a 5 min application of lidocaine-prilocaine cream (EMLA, AstraZeneca, Södertälje, Sweden), 1 ml of mepivacaine was infiltrated submucously and a punch biopsy with a diameter of 6 mm was obtained approximately 1-2 mm lateral to the orifice of the right Bartholin's gland. In 16 women not using COC, a corresponding biopsy was taken on the left side two weeks later (i.e. in the luteal phase) (Fig. 12). The biopsies were placed in a neutral buffered 4% formaldehyde solution for a maximum of 24 hrs. Dehydration, fixation and paraffin embedding were performed according to a standard protocol. Sections were cut perpendicularly to the surface, with a nominal thickness of 5 μ m, mounted on slides and stained with haematoxylin-eosin.

physical problems, Bodily pain and General health mainly represent physical health, while Vitality, Social functioning, Role limitations due to emotional problems and Mental health mainly account for the mental health. Health transition is an item measuring the subjective change in health rating during the previous 12-month period. A maximum score of 100 can be obtained for each item. SF-36 is widely used and has a high validity and reliability (Sullivan and Karlsson 1998). A study using the Swedish version of the questionnaire supports the cross-cultural stability of the ratings (Taft et al. 2004).

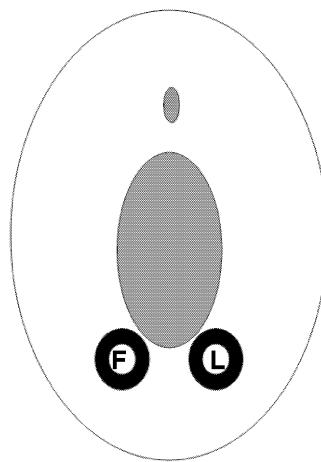


Fig. 12. Biopsy sites in the vulvar vestibule. F; 6 mm biopsy in the follicular phase. L; 6mm biopsy in the luteal phase.

4.5 MORPHOMETRY (III, V)

4.5.1 Epithelial thickness and morphology

A computer-assisted method was used to analyze the epithelial structure measuring the interdermal papilla distance, the distance from dermal papilla top to epithelial surface, distance from basal layer to epithelial surface and the dermal papilla width (Fig.13).

The specimens were placed in the microscope (Axioplan 2, Carl Zeiss, Jena, Germany) under the 5 X objective and an image of the specimen was captured in 8-bit RGB-mode into the computer (Sun SparcStation 20, Sun Microsystems Computer Corp., Santa Clara, CA) equipped with Micro-GOP 2000s, (Context Vision, Linköping, Sweden) image analysis software. All specimens were evaluated blindly.

By manually setting the threshold on the basal layer and thereafter removing larger subepithelial objects the epithelial profile is outlined and measurement operations begin

with the above described parameters (Blomgren et al. 2004). Every specimen was measured from the left side to the right with five equally spaced intervals of 411µm each.

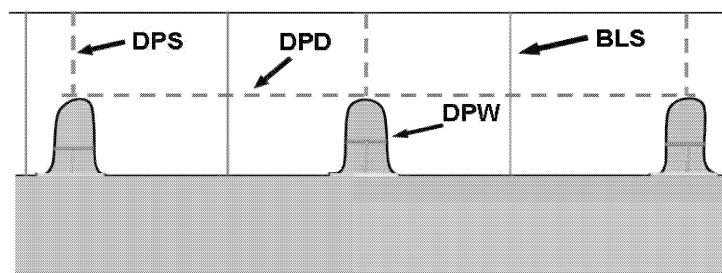


Fig. 13. Parameters measured in each specimen; DPD-interdermal papilla distance, DPS-distance from dermal papilla top to epithelial surface, BLS-distance from basal layer to epithelial surface and DPW-dermal papilla width.

4.5.2 Histopathological assessment

To further evaluate the morphology of the mucosa five related parameters were studied. The haematoxylin-eosin stained sections were coded and microscopically blindly evaluated by a pathologist regarding 1) cell distension 2) pycnotic nuclei 3) connective tissue density 4) superficial vessels in the connective tissue and 5) inflammatory cell infiltration in the connective tissue. A semi-quantitative estimation of each parameter was performed using a scale from 0-5 where 0 represents no sign and 5 major signs of the studied parameter.

The specimens were first scanned under low magnification, with the 5 X objective. This gave an overview of the specimen and allowed the determination of the connective tissue density as well as the infiltration of inflammatory cells. At higher magnification (20 and 40 X objective) the other parameters were determined. At 40 X magnification, the nature of the inflammatory cells could also be seen.

The rationale for studying the histopathological parameters was the fact that in the vaginal epithelium, cell distension along with pycnotic nuclei may indicate intracytoplasmic glycogen, a process which is estrogen induced (Speroff et al. 2005a). Furthermore, mucosal erythema observed in a subgroup of OC users with superficial

dyspareunia, could be explained by an increased superficial blood flow due to inflammation or an increased number of small vessels (arterioles and venules) in the upper part of the connective tissue. To rule out an ongoing cell mediated inflammation, the amount of lymphocytes in the connective tissue was estimated. The number of cell layers of the epithelium was also counted from the top of the dermal papillae to the surface at 5 locations in each specimen and a mean number of cell layers was calculated.

4.6 IMMUNOHISTOCHEMISTRY (IV, V)

4.6.1 Steroid receptors and Ki-67

A standard immunohistochemical technique (avidin-biotin-peroxidase) was carried out to visualize ER, PR, AR, GR and the proliferation marker Ki67 immunostaining intensity and distribution on the paraffin sections. Monoclonal mouse anti-human antibodies were used for detection of ER α (08-1149, 2nd Gen, Zymed Laboratories Inc, San Francisco, CA.), ER β (MCA1974, Serotec Ltd, Kidlington, UK.), PR-A (NCL-PGR 312, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), AR (M3562, DAKOCytomation, Carpinteria, CA) and Ki67 (NCL-Ki67-MM1; Novocastra Laboratories Ltd.). A monoclonal mouse anti-chicken antibody was used for PR-B (MA1-411, Affinity BioReagents (ABR), Golden, CO), and a polyclonal rabbit anti-human antibody was used for GR (PA1-511A, ABR). Antigen retrieval was performed by microwaving the sections for 20 minutes in 10 mM citrate buffer at pH 6.0. After washing in buffer (0.1M PBS, pH 7.4 for ER α , PRA, PRB, AR, GR and Ki67, and 0.1M Tris-buffered saline (TBS), pH 7.4 for ER β), non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 10 minutes at room temperature (RT). Following a 10 minutes wash in buffer, sections were exposed to a 30 (ER α , PRA, PRB, AR, Ki67), 60 (GR) or 45 (ER β) minutes non-immuno block using diluted normal goat serum (DAKOCytomation) in PBS (GR) or diluted normal horse serum (Vector Laboratories, Burlingame, CA) in PBS (ER α , PRA, PRB, AR, Ki67) or TBS containing 5% (w/v) BSA (ER β) in a humidified chamber at RT. The tissue sections were thereafter incubated with the primary antibody. The antibodies were diluted 1:5 (ER α), 1:500 (PR-A), 1:100 (PR-B and AR), 1:200 (Ki67), 1:1000 (GR) in PBS, and the ER β antibody was diluted 1:20 in TBS with 5% BSA. They were then incubated on sections overnight at +4°C (ER α , ER β , AR, GR, Ki67) or for 1h at RT (PRA, PRB). Following primary antibody binding, the sections were incubated for 30 (ER α , AR, Ki67), 45 (PRA, PRB) or 60 (ER β) minutes at RT with the second antibody, a biotinylated horse anti-mouse IgG (Vector Laboratories), diluted in normal horse serum. GR was incubated for 60 min at RT with the secondary antibody, a biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted in normal goat serum. Thereafter the tissue sections were incubated for 30 (ER α , ER β , PRA, AR, GR, Ki67) or 60 (PRB) minutes at RT with horseradish peroxidase-avidin-biotin complex (Vectastain Elite, Vector, CA). The antigen-antibody binding was detected with the application of 3,3'-diaminobenzidine (DAKO Cytomation, Carpinteria, CA, USA), a chromogen which produces a brown insoluble precipitate when incubated with enzyme. The sections were then counter stained with hematoxylin and analyzed with the use of light microscopy.

Negative controls were obtained by replacing the primary antibodies with an equivalent concentration of mouse IgG, except for GR where rabbit IgG was used.

4.6.2 Image analysis

The sections were placed under a Leica light microscope with a Sony video camera (Park Ridge, NJ, USA) connected to a computer with an image analysis system (Leica imaging system Ltd, Cambridge, UK). In this way we were able to assess semi-quantitative values from immunohistochemistry. In short, by using color discrimination software the total area of positively stained nuclei (brown color) was measured and expressed as the ratio of the total area of cell nuclei (brown + blue). The density of the positive staining was measured by three different color discriminations: strong (+++), moderate (++) or faint (+) brown reaction. Quantification of immunostaining was performed on the digitized images of systematic randomly selected fields of stroma, from which the epithelium was interactively removed and analyzed separately. All the epithelium, as well as 10 fields of vestibular stroma were measured separately in each tissue section. Sections from each part of the mucosa were analyzed by the same observer (epithelium UJ, stroma BM).

4.7 QUESTIONNAIRE (I-V)

A socio-medical profile was obtained by a standardized and coded questionnaire, see appendix on page 54. Results are shown in Table 3.

4.8 STATISTICS

Analyses of variance (ANOVA) were used in study I. The results of the mechanical and thermal stimulation were first analyzed with two-way ANOVA with repeated measurements, comparing the anterior and posterior areas within the two groups. Secondly, one-way ANOVA with post-hoc comparisons of means, the Tukey HSD test, was performed to compare the thresholds obtained in the anterior and posterior areas between the different groups.

Socio-medical differences between the groups were analyzed by the T-test (study I-V). Chi-square test was applied to detect differences in the number of high HADS scores. The score was dichotomized in high (8 and above) and low (<8) and the participants in patients and healthy women (study II).

The Kruskal Wallis ANOVA by ranks was used to detect differences between three groups regarding pain thresholds, VAS and differences in HADS scores (study II).

The SF-36 was analyzed using the Tukey-Kramer test (study II).

The Mann-Whitney U test was applied to detect differences between two independent groups, i.e. in pressure pain thresholds, in immunohistochemistry scores, in epithelial parameters and histopathological assessments (study II-V).

The Wilcoxon Matched Pairs test was used to detect differences within each group, i.e. in pain thresholds before and during cold stimulation, in immunohistochemistry scores, in epithelial parameters and histopathological assessments (study II-V).

The level of significance in all tests used was 0.05.

5 RESULTS

5.1 SOMATOSENSORY FUNCTIONS OF THE VESTIBULAR MUCOSA (I)

Twenty healthy COC-users and 19 nonusers underwent punctate mechanical and thermal stimulation. Significant differences in mechanical pain thresholds were observed. The results are presented in Figure 14. In all subjects, the posterior vestibule (area B) showed a tendency towards a lower pain threshold than the anterior part (area A). The COC users demonstrated hypersensitivity to punctate mechanical stimulation in area A, $p<0.05$. In area B the punctate mechanical threshold was significantly lower compared with non-COC users, $p<0.01$.

The results of the thermal stimulation showed no significant differences between the groups. The difference between detection of warmth and cold in area A was $10.2\pm1.1^{\circ}\text{C}$ in women on COC and $9.1\pm1.1^{\circ}\text{C}$ in non-COC users, ns. The heat pain threshold was $44.7\pm0.6^{\circ}\text{C}$ in area A and $44.8\pm0.7^{\circ}\text{C}$ in area B (ns) for the women on COC. The women not using COC had a heat pain threshold of $43.1\pm0.8^{\circ}\text{C}$ in area A and $44\pm0.9^{\circ}\text{C}$ in area B, ns.

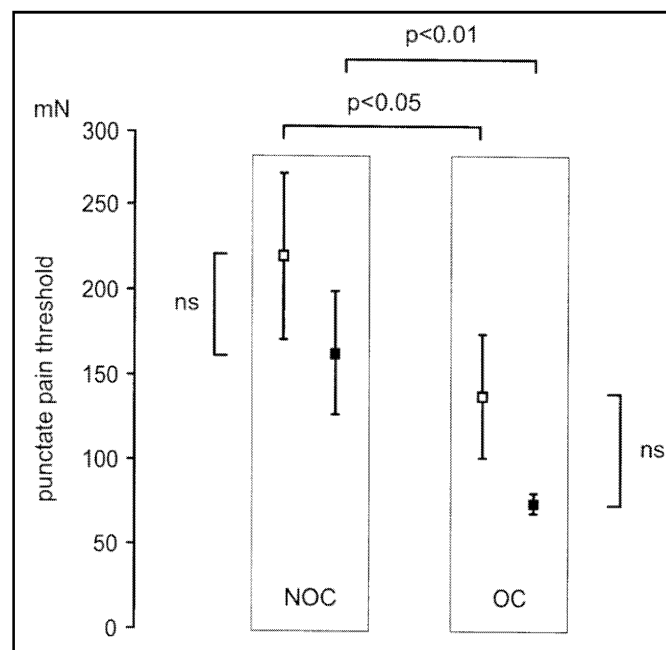


Fig. 14. Results from the punctate mechanical pain thresholds. Significant differences were obtained in both areas A and B between the two groups. NOC, non oral contraceptives; OC, oral contraceptives. Area A, open squares; area B, filled squares.

5.2 ENDOGENOUS PAIN MODULATION (II)

5.2.1 DNIC and pressure pain thresholds on the leg

All subjects showed significantly higher PPTs on the leg compared with the arm, both before and during the cold noxious stimulation.

During cold noxious stimulation there was a significant increase in PPTs within each group indicating a DNIC response. The patients increased their pressure pain thresholds from median 594 kPa (interquartile range 456-732) to 659 kPa (530-996), the non COC from 822 kPa (617-925) to 931 kPa (784-1291) and the COC from 974 kPa (764-1230) to 1105 kPa (876-1280), Fig. 15. Significantly lower PPTs were measured before cold noxious stimulation in the patients compared with the healthy study group, Fig. 15. During the cold noxious stimuli the PPTs in patients increased but were still lower than that of the COC group. However, there was no difference between the patients and women not using COC (Fig. 15). When comparing the healthy women (nonCOC and COC) with each other no significant differences were found in PPTs prior to and during the cold noxious stimulation.

Subtractions of the baseline PPT were made from the PPT during the cold noxious stimulation and a negative or zero score was considered as an absent DNIC response whereas a positive difference was considered a DNIC response (Edwards et al. 2003a). The subjects with a DNIC response were regarded as DNIC responders. DNIC responders on the leg were 16/20 patients, 16/20 in the non COC group and 13/20 in the COC group. There was no difference in the number of responders between the groups. After excluding the non DNIC responders, new analyses of PPTs were performed testing differences between the groups prior to and during the noxious stimulation. Subsequently only a difference in the patients compared with the COC group appeared. Prior to the noxious stimulation, median PPTs in the patients were 594kPa (456-752) and COC 870kPa (749-1188), $p=0.02$ in COC users and during stimulation 761kPa (577-1083) in the patients and 1124kPa (936-1292), $p=0.04$ in COC users. When comparing the non COC and COC groups, no significant differences were found regarding PPTs prior to or during the cold noxious stimulation.

5.2.2 DNIC and pressure pain thresholds on the arm

Within each group there was a significant increase in PPTs on the arm during cold noxious stimulation. The increase in the patients was from median 354 kPa (interquartile range, 260-463) to 377 kPa (309-365), in non COC users from 684 kPa (547-899) to 792 kPa (639-996) and in COC users from 660 kPa (487-906) to 769 kPa (559-990), Fig. 16. The patients reported significantly lower PPTs on the arm prior to cold noxious stimulation, compared with the non COC, $p<0.001$, and to the COC, $p<0.01$. During the cold noxious stimuli the PPTs in patients were significantly lower compared with those in non COC users ($p=0.001$) and to the COC group ($p=0.01$), Fig 16. Analyses of PPTs between the non COC and COC showed no differences prior to or during cold noxious stimulation.

On the arm the DNIC responders were 15/20 patients, 16/20 in the non COC group and 15/20 in the COC group (ns). After removal of non responders, significant changes were found in the patients both before and during noxious stimulation. In the patients, PPTs prior to stimulation were 354 kPa (234-580) as compared to the nonCOC group

638kPa (491-746), $p=0.04$, and to the COC group, 688 kPa (491-929), $p=0.009$. Similar results were seen during noxious stimulation with PPTs in patients 474kPa (312-765), versus 765kPa (671-876) in the non COC group ($p=0.008$) and 836kPa (710-1071) in the COC group ($p=0.02$). When comparing the healthy women after removal of the non responders, the COC group displayed a tendency towards higher PPTs on the leg before the cold noxious stimulation, $p=0.05$, but all other parameters were non significant.

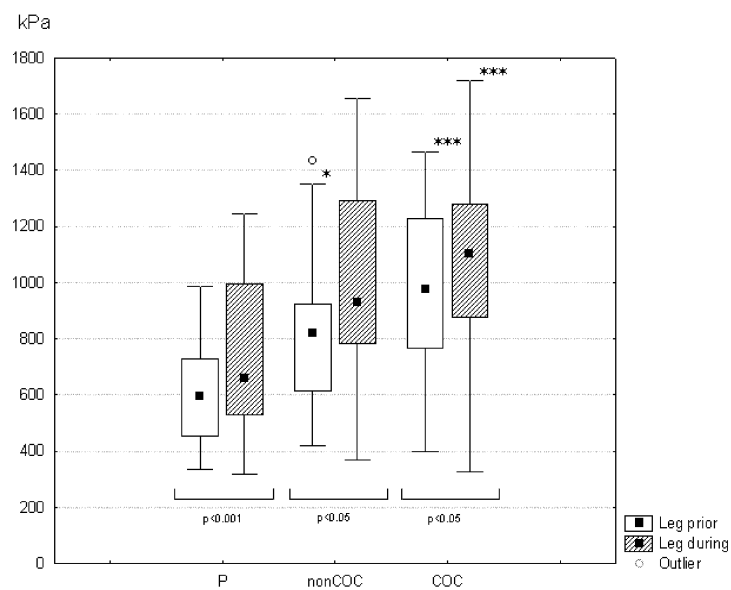


Fig. 15. Pressure pain thresholds in kilopascal (kPa) prior to (open boxes) and during (filled boxes) heterotopic noxious cold stimulation measured on the tibial muscle. ■ Median value, box: 25-75%, whiskers: non-outlier min – max. P= patients, nonCOC= healthy women without combined oral contraceptives, COC=healthy women using combined oral contraceptives.

* $p<0.05$, *** $p<0.001$.

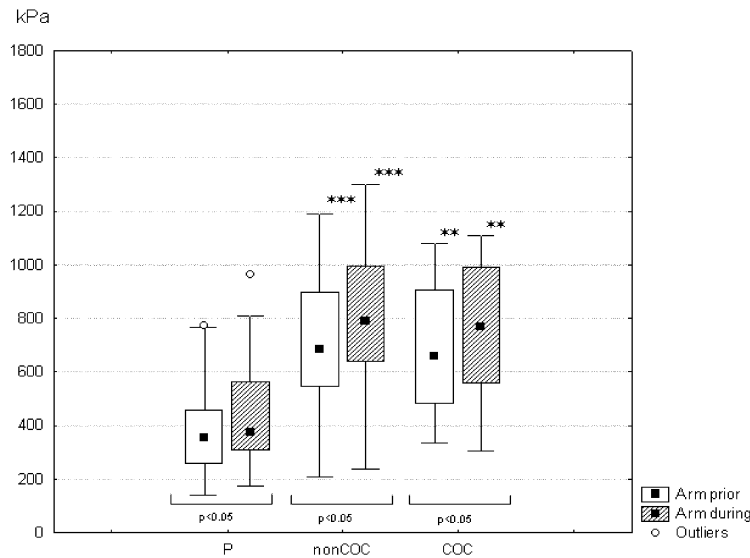


Fig. 16. Pressure pain thresholds in kilopascal (kPa) prior to (open boxes) and during (filled boxes) heterotopic noxious cold stimulation measured on the deltoid muscle. ■ Median value, box: 25-75%, whiskers: non-outlier min – max. P= patients, nonCOC= healthy women without combined oral contraceptives, COC=healthy women using combined oral contraceptives. ** p<0.01, *** p<0.001.

5.2.3 Concordance of DNIC nonresponders

Two patients were lacking a DNIC response on both the leg and arm compared with one non-COC user and three COC users. Among the remaining nonresponders the DNIC response was either lacking on the leg or on the arm.

5.2.4 VAS scores

Both groups of healthy women reported a significant increase in VAS scores while measuring the PPT on the leg during cold noxious stimulation, Table 5. No differences were seen in the patient group. There were no differences within the groups regarding the VAS scores on the arm. Comparisons between all groups before and during cold noxious stimulation showed no differences, Table 5. The VAS score for the cold pain stimulation of the immersed arm was significantly higher than the VAS scores for the PPTs on the leg and arm, Table 5, p<0.0001.

Table 5. VAS-scores.

Subjects	VAS leg (PPTs)	VAS arm (PPTs)	VAS cold pain
Patients (n=20)			
- prior stimuli	32 (19-58)	35 (23-50)	-
- during stimuli	46 (24-60)	41 (25-70)	78 (61-90)***
NonCOC (n=20)			
- prior stimuli	30 (24-53)	40 (25-60)	-
- during stimuli	36 (24-62)**	38 (24-65)	70 (58-83)***
COC (n=20)			
- prior stimuli	39 (18-55)	40 (20-61)	-
- during stimuli	48 (27-55)**	42 (22-53)	59 (48-83)***

** p<0.01 ***p<0.0001

5.3 HADS AND SF-36 (II)

The patients showed a higher score of anxiety in the HADS questionnaire, median 7 (iqr 5-10.5) as compared to the COC group 4 (1.5-6), p<0.01, and the non COC group 4 (2-5.5), p<0.01. The score for depression was also higher in the patients 4 (2.5-7) as compared to the COC group 0.5(0-2.5), p<0.01 and non COC group 1 (0-2), p<0.001. Nine patients out of twenty reported a high score (>8) on the anxiety scale compared to four out of forty of the healthy women, p<0.001. On the depression scale 3 patients reported high scores (>8) compared to none of the healthy women, p<0.05.

The SF-36 revealed a lower mean score for the patients on Vitality and General health compared to both the COC and the non COC groups, p<0.05, with no significant change in other items.

5.4 EPITHELIAL MEASUREMENTS IN THE VESTIBULAR MUCOSA (III, V)

5.4.1 Computer-assisted epithelial measurement

A larger distance between the dermal papillae was observed in biopsies from the COC group, median 543µm (iqr 305-765) compared with non COC users in the follicular phase 348µm (241-470), p=0.04 (Fig 17a). There was a larger distance from dermal papilla to surface, median 431µm (iqr 258-517) in COC compared with 287µm (217-

368) in nonCOC-follicular, $p=0.03$ (Fig 17b). No differences between biopsies from COC users and non users in the follicular phase were seen concerning distance of the basal layer to the surface or the width of the dermal papillae. The distance between dermal papillae was significantly larger in the luteal phase, median $571\mu\text{m}$ (interquartile range 384-817) than $348\mu\text{m}$ in the follicular phase of non users (239-403), $p=0.02$ (Fig 17a). There were no differences in the other parameters measured between the follicular and luteal phase.

There was a tendency, $p=0.06$, of a narrower width of the dermal papilla in the patients but no significant differences were found in the other epithelial parameters measured compared with controls, Table 6.

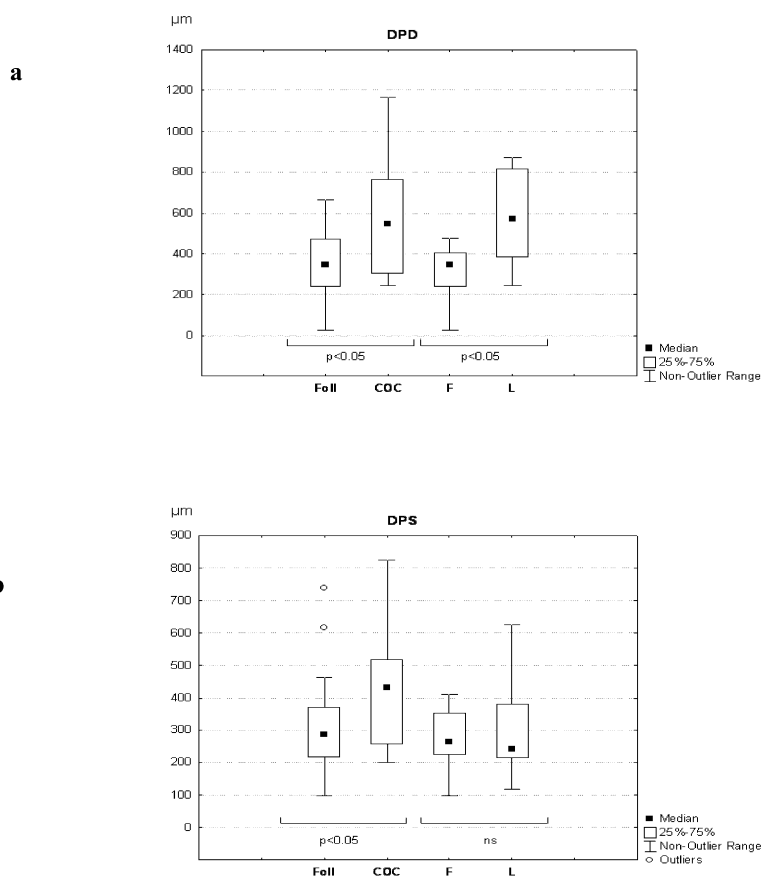


Fig 17 a and b. Biopsies were taken from 19 women using combined oral contraceptives (COC) compared to 24 women without COC in the follicular phase (Foll) as well as from 11 women without COC in both the follicular (days 7-11, F) and luteal phase (days 21-25, L). Figure a show significant differences in the distance between the dermal papillae (DPD) in μm between Foll and COC users as well as between F and L. Figure b show the significant differences between the COC users and Foll in the distance of the dermal papillae to the epithelial surface (DPS) in μm .

Table 6. Epithelial measurements in vestibulodynia patients compared to controls.

	DPD	DPS	BLS	DPW
Patients (n=13)	310 (237-433)	344 (224-422)	451 (388-523)	82^a (66-143)
Controls (n=24)	348 (241-470)	287 (217-368)	531 (430-598)	161 (77-199)

DPD - interdermal papilla distance, DPS - distance from dermal papilla top to epithelial surface, BLS - distance from basal layer to epithelial surface, and DPW - dermal papilla width. The distances are given in μm as median and interquartile range. There was a tendency ($\varnothing p=0.06$) towards a shorter DPW in the patients as compared to controls.

5.4.2 Histopathological assessment

A higher amount of small superficial vessels was observed in the connective tissue in the COC group (mean 2.68 and median 3, iqr 2-3) compared with the nonCOC during the follicular phase (mean 2.09 and median 2, iqr 2-3), $p<0.01$. For the nonCOC group a similar tendency was observed in the luteal phase (mean 2.27 and median 2, iqr 2-3), compared to the follicular phase (mean 2.09 and median 2, iqr 2-3), $p=0.07$. No other differences in cell distension, pycnotic nuclei, connective tissue density or inflammatory cell infiltration in the connective tissue were observed between the study groups. The subepithelial inflammatory cells observed were mostly lymphocytes. Our microscopical overview gave the impression of distended superficial cells in COC users.

The median number of cell layers was 15 (iqr 14-18) and 14 (12-18) in COC users and non users during the follicular phase, respectively. The number of cell layers did not differ between the follicular (median 18, iqr 14-20) and luteal phase (median 18, iqr 14-20) in nonCOC.

5.5 STEROID RECEPTORS IN THE VESTIBULAR MUCOSA (IV, V)

5.5.1 Serum estradiol and progesterone

Mean serum estradiol was 71 pmol/l (50-155) for the COC users and 310 (range 69-999) for the nonusers in the follicular phase, ($p<0.001$). Mean serum progesterone was 1.2 (0.5-3.3) for the follicular and 22 (13-37) nmol/l for the luteal phase.

Mean serum estradiol for the follicular phase was 244 pmol (range 118-393) in the patients as compared to 310 (69-999) in controls, ns. Mean serum progesterone was 0.85 (0.5-1.4) nmol/l for the patients as compared to 1.2 (0.5-3.3) nmol/l in the controls (ns).

All women reported regular menstrual cycles; however 5 samples were excluded from the paired analysis between the follicular and the luteal phase since the progesterone concentrations in the luteal phase did not indicate ovulation (<13 nmol/l). The results of the image analyses of the steroid receptors are shown in Figures 18 and 19.

5.5.2 Immunohistochemistry

5.5.2.1 Estrogen receptors

ER α expressing cells were distributed predominantly along the basal membrane and less frequently in stromal cells and vascular endothelial cells. The ER β positive cells showed a similar distribution but were more abundant in the stromal and vascular endothelial cells.

The total amount of ER β positive cells in the epithelium did not differ between COC users and nonusers (median 74 versus 68 %) (Fig.18).

The total area of ER β positive cell nuclei in the stroma was significantly larger in the COC group than in the controls (Fig. 19).

We calculated the ER α /ER β ratio in the stroma and epithelium in COC users and compared to nonCOC users in the follicular phase. The median ratio in the epithelium was 0.49 (iqr 0.22-0.84) in the COC group as compared to 0.64 (0.42-1.00) the nonCOC group, $p=0.08$. Thus, there is a tendency towards more ER β as compared to ER α in the epithelium in COC users. In the stroma the median ratio was 0.95 (0.77-1.42) in the COC users and 1.15 (0.92-2.04) in the nonCOC users (ns).

No significant differences were found regarding the estrogen receptor amount including ER α /ER β ratio between the follicular and luteal phases (F and L).

The expression of total amount ER α in both the epithelium ($p=0.04$) and the stroma ($p=0.02$) was higher in the patient specimens compared with those of healthy women in the follicular phase, Figures 18 and 19. There were no significant differences in the expression of ER β between the patients and controls.

5.5.2.2 Progesterone receptors

The PR α expressing cells were sparsely distributed along the basal membrane and more frequently in the stroma. No differences between groups were found (Fig 19).

Occasional PR β positive cells were found in the epithelium. Due to the scantiness it was not possible to perform an image analysis. Stromal cells expressing PR β were found and there was significantly less stromal PR β in the luteal phase (Fig. 19) compared with the follicular phase ($p=0.01$).

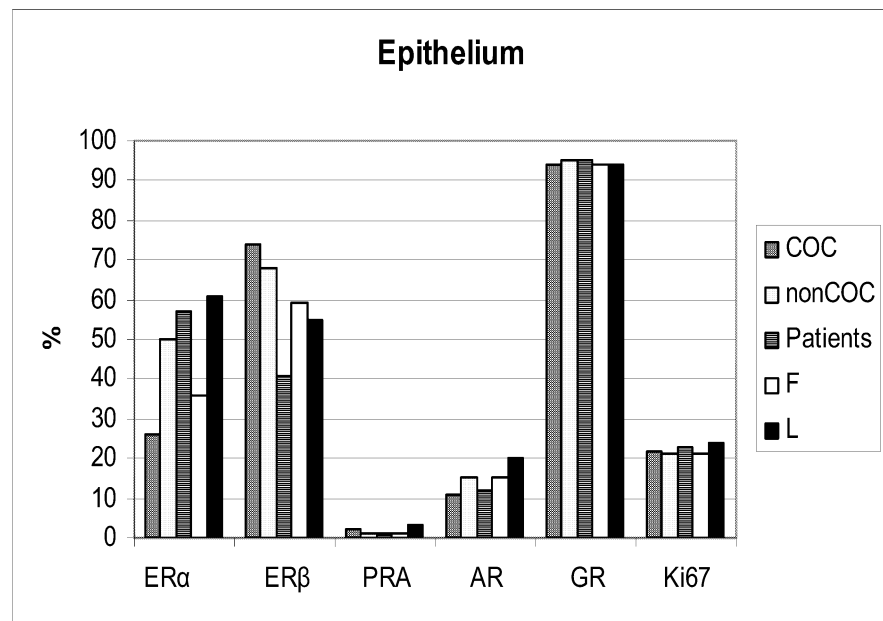


Fig. 18. Steroid receptor expression in biopsies from the vulvar vestibular epithelium. NonCOC = 25 women without combined oral contraceptives in the follicular phase, COC=20 women using combined oral contraceptives, 13 patients; and F and L = biopsies from 11 women analyzed in both the follicular and luteal phase.

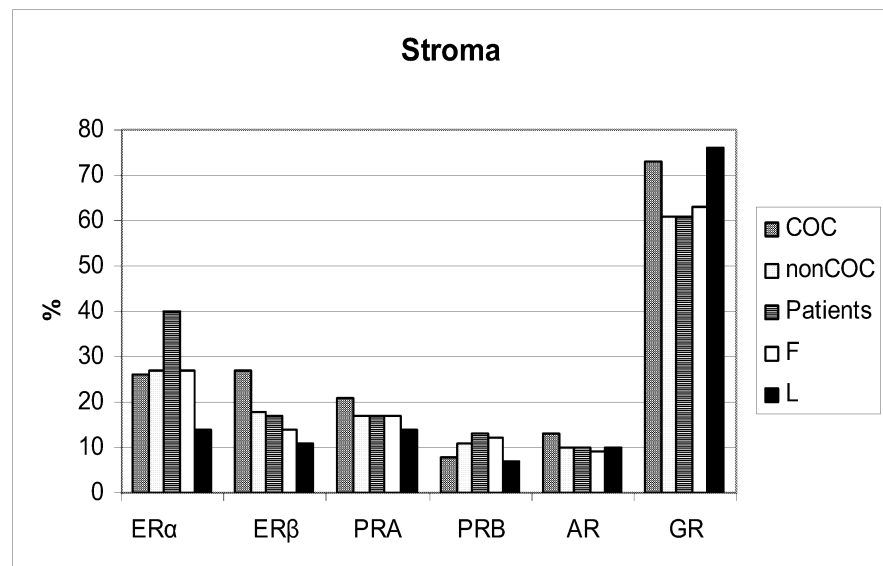


Fig. 19. Steroid receptor expression in biopsies from the vulvar vestibular stroma. NonCOC = 25 women without combined oral contraceptives in the follicular phase, COC=20 women using combined oral contraceptives, 13 patients; and F and L = biopsies from 11 women analyzed in both the follicular and luteal phase.

5.5.2.3 Androgen and glucocorticoid receptors

The AR expressing cells were found in the suprabasal part of the epithelium as well as in the stroma. No differences between the groups were found.

In the basal and suprabasal part of the epithelium 94-95% of the cells and 61-75% of the cells in the stromal tissue including vascular endothelial cells were positively stained for GR. There were no differences between groups (Fig. 18 and 19).

5.5.2.4 Ki67

Ki67 is a nuclear antigen expressed in cells during proliferative activity and largely restricted to basal and parabasal epithelial cells in normal tissues (van Hoesen and Kovatich 1996).

We found Ki67 expression in the basal part of the epithelium. There were no differences between COC users and nonusers and no variation during the menstrual cycle (Fig.18). There were no significant differences between patients and healthy women in the follicular phase (Fig.18).

6 DISCUSSION

6.1 DISCUSSION OF THE MATERIALS AND METHODS

6.1.1 Participants

All the vestibulodynia patients were recruited from the Vulvar Clinic at Danderyd Hospital, whereas the healthy women were recruited through advertisements at Karolinska Institutet and Stockholm University. A selection bias must be considered since the majority of the healthy participants were university students. However, among the patients there was also a predominance of subjects with a high education thus matching the controls. The control subjects were of the same age range as the patients and were easy to recruit. The higher level of education could result in individuals being more interested in research and willing to participate compared with women with a lower educational degree.

6.1.2 Quantitative sensory testing

QST is considered as the only clinical test available to quantitatively assess the function of somatic small nerve fibers (Yarnitsky et al. 1999). There is a lack of psychophysical studies evaluating sensory functions in the vulvar region in healthy individuals (Eva et al. 1999) and there are limited data on normal values for temperature and pain thresholds to sensory stimulation in this area. Consequently, paper I can be regarded as a descriptive study on vulvar sensory perception in healthy young women serving as reference data for future research. For practical reasons we used the method of limits, which includes the subject's reaction time. Using this method the sensory thresholds are affected by the reaction time, which is not included in the methods of levels (Yarnitsky and Ochoa 1991).

6.1.3 Pressure pain thresholds and pain modulation

Methodological aspects on the testing procedures should be considered. Pain perception may differ throughout the menstrual cycle and the testing was therefore performed days 7-11 after the onset of the last bleeding to standardize for any difference related to the menstrual cycle (Riley et al. 1998; Fillingim and Ness 2000). The PPTs were assessed manually and much effort was made to hold the pressure algometer perpendicularly to the skin and avoid an "edge-effect" which might influence the result. A digital display was also used in order to control the pressure speed to the best ability. However, the wide interindividual variation of the PPT results might reflect an inconsistency in the method used. The pain thresholds on the upper arm were lower in all groups compared to the thresholds on the leg. This finding was anticipated since the tested area of the deltoid muscle is a well known trigger point in contrast to the anterior tibial muscle. Furthermore, the higher VAS score for the cold pain in all groups compared to VAS for the pressure pain on the leg and arm indicate that the heterotopic noxious stimuli was sufficient to induce a DNIC response. However, a limitation to the study is the lack of a control tank with 22°C water permitting the exclusion of confounding results from pure water immersion. The healthy women displayed an increase in the VAS scores on the leg before and during cold noxious stimulation in contrast to the arm and compared to

the patients. This increase remains an enigma. A plausible explanation could be the initial low level of anxiety as indicated by the HADS questionnaires in the healthy individuals and the possible anxiety increase and discomfort during cold water immersion affecting the subjective VAS rating. The healthy women received a higher level of pressure pain stimulation than the patients which also might influence the VAS scores.

6.1.4 HADS and SF-36

We have only used validated questionnaires, modified for use in Sweden. When collecting clinical data through a questionnaire one must always bear in mind cultural differences which might influence the results. Since both HADS and SF-36 has been used in several studies on chronic pain patients, we regard these as reliable methods (Bjelland et al. 2002; Snaith 2003; Taft et al. 2004).

6.1.5 Epithelial thickness and histopathological assessment

Several methods have been used in studies evaluating the morphology of genital mucosa. Usually the epithelial thickness has been defined as the length from the basal membrane to the epithelial surface in μm and structural variations of the dermal papillae have not been taken into consideration (Chiang et al. 1998; Mauck et al. 1999). A computerized method has recently been developed to achieve a more unbiased epithelial measurement (Blomgren et al. 2004). We expected to obtain a more accurate image of the epithelial profile by measuring four different parameters. Previously only minor cyclic variations in the vaginal mucosa have been observed, probably due to methodological differences (Patton et al. 2000).

By electron microscopical analyses, ultrastructural changes in EMLA treated skin biopsies have been reported (Vallance et al. 2004). We used light microscopy and were not able to detect any abnormalities. EMLA cream was applied similarly in the same way in all subjects, thereby minimizing potential differences between the groups regarding vasculature and epithelial thickness (Egekvist and Bjerring 2000; Hafner et al. 2003).

6.1.6 Immunohistochemistry

We present the expression of several steroid receptors in the vulvar vestibular mucosa. Such a comprehensive study has previously not been published. We tried to avoid bias by blinded analysis of the specimens using computerized methods. One limitation of the immunohistochemical analysis study is the lack of quantification by polymerase chain reaction (PCR) and/or Western blot. Such analyses would have required larger biopsies, which out of an ethical point of view was refrained from in this very sensitive area.

6.2 DISCUSSION OF THE RESULTS

6.2.1 Peripheral and central pain mechanisms

There are many ways to evaluate peripheral and central pain mechanisms. In the first study we have focused on the peripheral sensory perception in the vulvar vestibule in healthy women with and without COC. In the same study it would have been

interesting to extend the QST and measure pain thresholds on other parts of the body of the participants. Instead, we performed a sequel study using a pressure algometer to measure general PPTs before and during cold noxious stimulation in COC users, nonusers and in patients with provoked vestibulodynia.

6.2.1.1 Decreased vestibular mechanical pain thresholds in healthy women using COC

The lower pain thresholds registered in the vulvar vestibulum in COC users may represent a subclinical allodynia which might be explained by structural changes with unaffected nerve fibers in the vestibular mucosa. During prolonged progestin influence, the endometrium and vaginal mucosa will become atrophic (Sjoberg et al. 1988; Hild-Petito et al. 1998; Benagiano et al. 2004). In another study on vagina however, the authors found a hyperplastic epithelium in women using depot medroxy-progesterone-acetate injections (Ildgruben et al. 2003).

If COC are inducing morphologic alterations in the vestibular mucosa, probably the mechanical properties and thus the conduction of mechanical stimuli to the nerve receptor will be affected. These speculations are supported by the observation of an increased relative risk of provoked vestibulodynia when COC with high progestogenic potency are used (Bouchard et al. 2002).

In case of a thinner epithelium it is reasonable to assume that pain-conducting nerve endings are more superficially located causing lowered threshold when mechanically stimulated. However, also a thicker epithelium might interfere with the pain transmission.

The mucus secretion from the vestibular glands may act as a shield for the epithelial surface (Sargeant et al. 1996). Women on COC may interrupt the use of the pill due to vaginal dryness affecting the sexual life (Sabatini and Cagiano 2006). The enhanced sensitivity in the vestibular mucosa that we observed in healthy COC users might thus in part be related to a diminished amount of protective mucus. In a study dealing with ocular dryness it was found that a reduced number of goblet-cells on the inferior bulbar conjunctiva were significantly correlated to COC use (Connor et al. 1999).

Central pain mechanisms may also be affected by COC. Pain thresholds to various stimuli have been found to be lower in healthy COC users compared with nonusers and men (Bragdon et al. 2002; Kowalczyk et al. 2006). Estrogen receptors in enkephalin-producing neurons in the spinal cord have been observed in rats (Amandusson et al. 1996). After injections of estrogen benzoate the levels of preproenkephalin mRNA were increased, suggesting estrogen as a potential endogenous pain modulator (Amandusson et al. 1999). It is still however, unclear if COC per se can alter the central pain transmission.

Progesterone may convert into neuroactive metabolites, a process which can be inhibited by substance P, suggesting a link between nociception and progesterone (Patte-Mensah et al. 2005, 2006). A progesterone receptor antagonist has been proven efficient in relieving neuropathic pain, further emphasizing an association between pain and hormones (Kondo et al. 2006).

6.2.1.2 Decreased general pressure pain thresholds in women with provoked vestibulodynia

Prior to the cold noxious stimuli, general pressure pain thresholds (PPTs) were lower in the vestibulodynia patients than in the healthy women irrespective of COC status. This phenomenon in the patients implies the presence of an altered central sensory processing of the pain transmission. The result is in accordance with that of other studies (Pukall et al. 2002; Giesecke et al. 2004; Pukall et al. 2005). It has been suggested that women with primary provoked vestibulodynia have a dysfunctional central pain modulation in addition to the vestibular pain. This could partly explain their poor treatment outcomes (Granot et al. 2004a, b). These findings support the hypothesis of certain alterations in the central nervous system in this group of women.

Compared with the healthy women, the patients also reported more general pain manifested as headache, muscle ache, low back pain and dysmenorrhea. Since one exclusion criteria for the healthy women in this study was frequent medication with analgesics there might have been a bias in the selection of the control subjects. Nonetheless, this was taken into consideration while designing the study. Two strong confounding factors were avoided by excluding control subjects with chronic pain and ongoing depression requiring medication. Since a deficient endogenous pain modulation has been observed in chronic pain patients it was important to exclude such subjects (Lautenbacher and Rollman 1997). In addition, frequent bodily pain has also previously been reported in patients with vulvodynia, suggesting a deficiency in pain modulation and a current central sensitization (Danielsson et al. 2000; Harlow and Stewart 2003).

A genetic predisposition for increased pain sensitivity has been found. Individuals carrying a pain-protective haplo-type for GTP cyclohydrolase were significantly less sensitive to mechanical pain and tended to be less sensitive to heat and ischemic pain (Tegeder et al. 2006). Genetic variants with low enzymatic activity of catecholamine-O-methyltransferase have also been suggested as having a high risk of developing chronic pain conditions (Diatchenko et al. 2005; Nackley et al. 2007). Certain genetic profiles seem to be correlated to provoked vestibulodynia (Witkin et al. 2002; Gerber et al. 2003; Foster et al. 2004).

6.2.1.3 Pressure pain thresholds, DNIC and COC

During the induced cold pain there was a significant increase in PPTs for all groups tested, but less prominent for the healthy women not using COC. After exclusion of the DNIC nonresponders it was found that the PPTs on the leg before and during cold noxious stimulation were higher in oral contraceptive users than in the patients. In contrast to our results other authors (Bragdon et al. 2002; Kowalczyk et al. 2006) found lower pain thresholds in healthy COC users. Another study evaluating sex differences in DNIC revealed lower VAS pain scores in women on OC compared with women without OC and men (Baad-Hansen et al. 2005). Provoked vestibulodynia may be regarded as a regional pain condition and resembles temporomandibular disorder (TMD). It has been shown that women using COC have an increased risk for TMD (LeResche et al. 1997). The lack of differences between the healthy women in the

present study is compatible with a peripheral component when speculating whether COC is a contributing factor for the development of vestibulodynia.

6.2.1.4 DNIC

The majority of women in all three study groups had a DNIC response, i.e. increased mechanical pain thresholds elicited by cold noxious stimulation. Previous studies on the DNIC function in different pain conditions show divergent results. Patients with rheumatoid arthritis (RA) display a preserved DNIC function (Leffler et al. 2002b) whereas a deficiency has been found in fibromyalgia (FM) patients (Lautenbacher and Rollman 1997; Julien et al. 2005). In more localized pain conditions such as trapezius myalgia (TM) the patients display intact DNIC-related mechanisms (Leffler et al. 2002a) in contrast to TMD and chronic tension-type headache (CTTH) patients in whom a deficiency in these endogenous pain inhibitory mechanisms has been reported (Maixner et al. 1995; Kashima et al. 1999; Pielsticker et al. 2005). These observed alterations in DNIC responses imply that endogenous pain modulation is a dynamic function and not only related to whether a pain condition is regional or generalized, but most probably also if the pain is intermittent or more continuous. Women with vestibulodynia suffer from provoked and intermittent pain with pain-free intervals in contrast to TM, RA and FM, characterized by long-term and more or less ongoing chronic pain. Maybe the explanation for the difference in DNIC function between TMD and vestibulodynia can be sought in the frequency of the pain triggering mechanism; chewing versus coital provocation? It was recently observed that women treated for vestibulodynia displayed lower general pressure pain thresholds while the vestibular pain thresholds were significantly increased after treatment. However, in spite of the reported subjective improvement of coital pain, the vestibular pain thresholds were still lower than in healthy women (Bohm-Starke et al. 2007, in press).

The DNIC response is influenced by age (Edwards et al. 2003a) and may be reduced or absent in older people. In our study, the numbers of DNIC responders as well as the age span of the participants were similar in each group. Women with fibromyalgia are older compared with women with provoked vestibulodynia. Is it plausible that the lack of DNIC response in fibromyalgia may be explained by the age of the patients? There might be a gradual decrease in endogenous pain modulation over time. It would be interesting to know whether women with provoked vestibulodynia will develop other pain syndromes later in life. An association between vulvodynia and other co-morbid conditions such as fibromyalgia (OR 3.84) and irritable bowel syndrome (OR 3.11) (Arnold et al. 2006) has been reported. The DNIC response has also been suggested to be a variable process. In a study on osteoarthritic patients the DNIC response was absent before hip surgery but it reappeared 6-14 months after the surgery (Kosek and Ordeberg 2000).

Placebo analgesia has been considered mainly as a cortical phenomenon. However, Goffaux et al. studied the DNIC system in two groups of participants; the first were told that the procedure would produce analgesic effects. In the second group, expecting the procedure to be pain-enhancing, the DNIC effect (i.e. analgesia) was completely blocked (Goffaux et al. 2007).

In this study we used thermal noxious stimuli to evaluate the supraspinally mediated endogenous pain control in women with vestibulodynia. The cold pressor test will mainly affect C-fiber mediated pain and is regarded more effective than tonic heat as a DNIC inducer (Price and McHaffie 1988; Watanabe et al. 1996). For a deeper understanding of other noxious modalities, the tourniquet (Bouhassira et al. 2003) and hypertonic saline injections (Ge et al. 2004) could be used as a DNIC inducer.

The current opinion on endogenous pain modulation includes both facilitating and inhibitory factors (Gebhart 2004). We have only studied one pain inhibitory mechanism and consequently we would also need to evaluate descending facilitating mechanisms which may dominate in our patients (Zhao and Gebhart 1992).

6.2.2 Anxiety and depression in provoked vestibulodynia

It is well known that the psychological condition of a subject will have an influence on the pain perception (Thompson et al. 2007). The anxiety and depression reported by more patients than controls might be explained by the long-lasting dyspareunia (5.7 years). The result is compatible with those of other studies (Sackett et al. 2001). In one study on vulvodynia, the life time prevalence rate for major depressive disorder was 45%. The majority of patients had their first depressive episode before the onset of vulvodynia (Masheb et al. 2005) and a similar finding was reported in another Swedish study (Nylanderlundqvist and Bergdahl 2003). However, from our material we cannot reveal whether the patients were more prone to mood disorders compared with healthy controls, i.e. if an underlying mood disorder preceded the vestibulodynia.

6.2.3 The vulvar vestibular mucosa- morphology

Our results show an altered morphological pattern with low and sparse dermal papillae in healthy young women using COC compared with the situation during the follicular phase in women without COC. A similar variation in the morphology was also found during the menstrual cycle with an increased distance between the dermal papillae in the luteal phase.

Our results are in concordance with Ildgruben et al. who also found a thicker vaginal epithelium under usage of COC (Ildgruben et al. 2003). According to another study, the vaginal epithelium was unchanged during usage of COC (Eschenbach et al. 2000). In macaques it has been shown that the vaginal epithelium will become extremely thin during the influence of exogenous high dose progestins (Hild-Petito et al. 1998).

We have evaluated the vulvar epithelial morphology by four different parameters and the number of epithelial cell layers was estimated. We did not find any difference in the number of cell layers during COC use or during the menstrual cycle. However, in spite of a similar number of cell layers, there was a greater distance from the dermal papillae to the surface in the COC group. This phenomenon may be due to the presence of larger keratinocytes. The histopathological assessments did not disclose any differences in cell distension or nuclear size. Nevertheless, during the microscopical overview, the superficial cells in COC users appeared distended supporting previous findings in the vaginal epithelium (Ildgruben et al. 2003). In addition, a low karyopycnotic index has been observed in the vaginal epithelium of women using oral contraceptives (Katira and Dayal 1987).

In one study, a minor reduction of vaginal cell layers was observed in the luteal phase whereas no cyclic variation was found in another (Ildgruben et al. 2003; Patton et al. 2000). The luteal phase, and its morphological changes, is influenced by serum progesterone (Li and Cooke 1991). The mucosa might respond differently to endogenous sexual hormones or to exogenous progestins in combination with 17-ethinyl estradiol. Since the morphology of the vulvar vestibular mucosa was comparable in COC users to that during the luteal phase, the effect is most likely due to progestins. Possibly the use of COC will induce morphological qualities making the mucosa more vulnerable to mechanical strain.

The basal membrane zone (BMZ) is a forceful link between the epithelium and the underlying connective tissue. The basement membrane is wrinkled by interlocking the downward projection of the epithelium with the dermal papillae. This profile is specific for different body regions (Briggaman 1982). Several dermatoses that may affect vulva are causing structural changes of the epithelium (Ball and Wojnarowska 1998). Psoriasis is associated with an epidermal proliferation creating a characteristic morphologic pattern with an increased number and height of dermal papillae (Iizuka et al. 2004). In lichen sclerosus there are a lower number of dermal papillae which also are shallower (Kowalewski et al. 2005). In erosive lichen planus, the BMZ is disrupted in affected mucosal lining causing a very fragile mucosa (Ball and Wojnarowska 1998). It may be speculated whether during progestin influence, shallow and more separated dermal papillae will affect the interlocking function of the dermal papillae making the mucosa more vulnerable? High dermal papillae are found in areas exposed to an enhanced mechanical stress, such as the palate (Klein-Szanto and Schroeder 1977). In aging skin a flattening of the dermal-epidermal junction is seen causing a more fragile barrier (Lavker et al. 1987).

Our earlier observation of an erythematous vestibular mucosa in the COC users could perhaps be explained by an enhanced number of small vessels. An evaluation of the superficial blood flow would also add important data to this finding. It has been observed that the vascular topography and permeability of gingival tissue may be altered by OC (Vennetti and Miller 1987). An increased gingival inflammation has also been reported in COC users (Kalkwarf 1978). The vestibular and gingival mucosa have many features in common (Heilman 1987). Progesterone and estrogen receptors are found in the vulvar epithelium although more sparsely than in the endometrium and the vagina (Hodgins et al. 1998). Estrogen receptors have been found in the endothelium of blood vessels in various tissues supporting hormonal influence on vascularization (White 2002). If COC use really affects the morphology, the epithelium may require an elevated nutritive blood supply. Such a situation might be another reason to the increased vascularization observed in the vulvar vestibular tissue of COC users.

In women with provoked vestibulodynia there was a tendency to a narrower dermal papilla width compared with healthy controls (Table 6). If this is an accurate situation, it could also affect both the interlocking function between epidermis and dermis. This might be a residual sign of a dysfunctional epithelium under repair. As earlier mentioned, the dermal papillae contain blood vessels and nociceptive afferents that will penetrate with free nerve endings into the epithelium.

6.2.4 The vulvar vestibular mucosa - steroid receptors

In this study we have shown that the vulvar vestibular expression of certain steroid hormone receptors varies during the menstrual cycle and seems to be influenced by contraceptive hormones. The vestibular stroma of healthy women on COC is expressing more ER β than that of control women during the follicular phase. One interpretation of these findings may be that ethinyl estradiol has a strong affinity to ER α but a much weaker affinity to ER β . Such a phenomenon has been observed in human cell lines (Barkhem et al. 1998; Escande et al. 2006). A possible compensatory mechanism reflected as an up-regulation of ER β might then be activated in the stromal tissue. Alternatively, the expression of the estrogen receptors might be influenced by the low endogenous 17 β -estradiol concentration or by the progestins in the combined oral contraceptives. Injections of depot medroxyprogesterone acetate did elevate the ER expression (Ildgruben et al. 2003). An increase in ER β has been demonstrated in the endometrium whilst the serum progesterone rises in the luteal phase (Taylor et al. 2005).

The ratio between the estrogen receptors α and β is believed to have a role for various tissue effects. Multiple functions have been associated to the presence of the estrogen receptor β (Koehler et al. 2005). In mammary gland tissue and probably also in the endometrium and myometrium ER β has got an anti-proliferative and pro-differentiative effect (Koehler et al. 2005; Hartman et al. 2006). Some studies imply a role between ER β and proteases involved in the destruction of extracellular matrix, with possible effects on endometriosis and pelvic organ prolapse (Ewies et al. 2003; Ewies et al. 2004; Pilka et al. 2004; Hudelist et al. 2005). An increased ER β expression has been shown in smooth muscle cell hypertrophy in male varicose veins (Knaapen et al. 2005). Our observation of ER β being mainly present in the parabasal layer of the epithelium and in endothelial cells is in agreement with earlier findings (Barchiesi et al. 2004). In the vestibulum of COC users there are more superficial blood vessels and consequently more endothelial cells, which predominantly express ER β . The link between an altered morphology and higher amount of ER β is more complicated. It seems as if COC not only will affect the expression of ER β but also the morphology of the vestibular mucosa. Stromal estrogen receptor α has been proven important for the growth of the vaginal epithelium. However, from other tissues we learn that the function of the steroid receptor can differ (Koehler et al. 2005). Since we found lower mechanical pain thresholds in healthy COC users than in nonusers, we are tempted to believe that the gestagenic influence is contributing to a more sensitive vulvar mucosa. In a retrospective study of 124 pre-menopausal women with sexual dysfunction the women who had never used COC reported less sexual pain compared with those who had used COC (Panzer et al. 2006).

We found a cyclic variation in PRB in the vulvar vestibular stromal tissue in women not using COC. The lower concentration of PRB in the luteal phase compared with the follicular phase might be due to a down regulating effect of progesterone (Li and Cooke 1991). On the other hand, in the uterine stroma of rats treated with estradiol during the last 24 hours before sacrifice, the PRB level was increased, while progesterone treatment on its own did not cause a significant increase of PRB positive cells (Sahlin et al. 2006). It is likely that the high estradiol level induce the increased PRB level during

the follicular phase. Our results are in accordance with those of endometrial PR (Mote et al. 1999; Noe et al. 1999). Vaginal PR was markedly down regulated by administration of depot-medroxyprogesterone acetate, but no variation was seen during the menstrual cycle or under influence of COC (Ildgruben et al. 2005). Stromal PR is known to crosstalk with other receptors in the epithelium. As shown in rats, such interactions may be regulated by estradiol and progesterone, where ER β signals will down-regulate the epithelial PRB mRNA levels (Sahlin et al. 2006).

The lack of cyclic variation in the other steroid receptors present in the vulvar vestibular tissue is consistent with studies on the vagina (Perez-Lopez et al. 1993; Ildgruben et al. 2005). However, in the endometrium, ER and AR as well as the Ki67 expression will vary during the menstrual cycle (Snijders et al. 1992; Perez-Lopez et al. 1993; Mertens et al. 2001; Taylor et al. 2005).

Progestins such as desogestrel and gestodene have shown an affinity to the GR (Fuhrmann et al. 1995) and the majority of progestins are able to bind to the AR (Garcia-Becerra et al. 2004). Cortisone as well as androgen treatments for various vulvar diseases have often been used (Haefner et al. 2005). COC will suppress the endogenous 17 β -estradiol synthesis and increase the level of sexual hormone binding globuline (SHBG) resulting in a lower free testosterone concentration (Panzer et al. 2006). Our study did not reveal any significant differences in the distribution of neither AR nor GR in COC users compared with the follicular phase or between women without COC during the menstrual cycle.

Our results do not support that of earlier findings of a lower level of ER α in patients with provoked vestibulodynia compared with control subjects. However the control material as well as the methodology differs between the studies (Eva et al. 2003). Our computerized receptor analysis of both the epithelium and stroma highlights the role of stromal ER α in epithelial morphology (Buchanan et al. 1998).

The increased expression of ER α could be due to a regenerating tissue after an earlier damage. However, there were no differences in the expression of the proliferation factor Ki67 in patients compared with controls. Although the epithelial morphology was similar to that of healthy pain-free women it still might be dysfunctional in the patients.

Despite conflicting results of histopathological studies, the prevailing theory is that cell-mediated inflammation is not involved as an etiological cause in women with provoked vestibulodynia (Lundqvist et al. 1997). There was no difference in the expression of GR between patients and controls, which might further exclude the role of cell-mediated inflammation. Consequently, there is no benefit from corticosteroid treatment in these patients (Haefner et al. 2005). Our findings support earlier studies on the lack of inflammatory markers in vestibular mucosa in the patients (Bohm-Starke et al. 2001a).

An increased blood flow and nociceptor sensitization has been found in the vestibular epithelium, supporting the theory on a present neurogenic inflammation in these patients (Bohm-Starke et al. 2001c). The increased amount of ER α could be related to the reported increase of peripheral free nerve endings in women with provoked vestibulodynia (Westrom and Willen 1998; Bohm-Starke et al. 1998). In a study on mouse bladder it was suggested that ER has a modulatory function through a variety of mediators engaged in neurogenic inflammation (Bjorling and Wang 2001). ER α has been found in sensory neurons with a small cell body diameter in the dorsal horn of

rats. This finding suggests that nociceptive afferents are expressing ER α (Taleghany et al. 1999). Animal studies have shown that ER subtypes are of importance for the vanilloid receptor function (Schroder et al. 2003). The vanilloid receptor is expressed by nociceptive fibers and is triggered by various noxious stimuli, such as capsaicin, with subsequent release of neuropeptides which are capable to induce neurogenic inflammation (Caterina et al. 1997). An increase of the vanilloid receptor VR 1 has been observed in vestibular biopsies from patients with provoked vestibulodynia (Tympanidis et al. 2004). To elucidate a correlation between vestibular ER α and neurogenic inflammation further studies are needed.

6.3 TREATMENT IMPLICATIONS

Since the etiology of provoked vestibulodynia is considered multifactorial, the treatment modalities vary. The histopathological and pain modulation results of our studies have given some suggestions to further treatment.

Topical estrogen have previously been tried, both with estradiol and estriol, however no studies have been published in this matter (Haefner et al. 2005). Our findings of an increased expression of ER α could implicate that topical estrogen have a role in the treatment. A double-blind, randomized and controlled trial is required.

Our earlier clinical observation of an erythematous and hypersensitive mucosa in healthy COC users may indicate that it is of importance to further elucidate the influence of hormonal contraceptives in the development of long-lasting vulvar pain. According to our experience, the women with dyspareunia who were cured or improved after an interruption of COC, were younger and had a shorter history of pain than women with provoked vestibulodynia. They might have shown early manifestations of vestibulodynia that could have resulted in prolonged and severe dyspareunia if not attended to. Doctors and midwives responsible for prescription of COC should be aware of these potential adverse effects on the genital mucosa. If vulvovaginal dryness, soreness or painful intercourse frequently will occur, an interruption of COC may be recommended. In that case, it is mandatory to advice of another contraceptive method.

Misoprostol is an antiprogesterone mainly used as a medical abortion pill. Several studies have observed that misoprostol also is effective as contraception (Narvekar et al. 2004; Narvekar et al. 2006). In a recently published study the vaginal thickness and steroid receptor content did not change after treatment with mifepristone, suggesting a lesser impact on the vaginal mucosa (Narvekar et al. 2007). It would be interesting to further evaluate this contraceptive method and the effects on the vulvar vestibular mucosa in women with vestibulodynia.

6.4 FUTURE PERSPECTIVES

A predisposition for increased pain sensitivity has been found in genetic studies. Individuals carrying a pain-protective haplotype were significantly less sensitive to mechanical pain and tended to be less sensitive to heat and ischemic pain (Tegeder et al. 2006). Some studies have implicated that certain genetic profiles are correlated to provoked vestibulodynia (Witkin et al. 2002; Gerber et al. 2003; Foster et al. 2004). Further studies on the genetic pain modulation profile in these patients are needed. It would also be interesting to follow women with vestibulodynia in long term studies to see if they develop other pain syndromes (e.g. fibromyalgia, TMD) later in life.

In this thesis only combined oral contraceptives are evaluated. New progestin-only contraceptive methods have been introduced in Sweden recently. A large epidemiological study with randomization between COC and progestin-only methods may further answer the question whether the influence on the vulvar vestibular mucosa is due to the progestins solely.

The vulvar clinic at Danderyd hospital has a long tradition in treating women with vulvar pain. Over the years a large database on women with provoked vestibulodynia and on healthy women in the same age group has been collected. A prospective follow-up of these subjects may contribute to a deeper knowledge on the natural lapse of vestibulodynia of which little is presently known.

7 CONCLUSIONS

Healthy women on COC display a higher vestibular sensitivity to mechanical pain stimulation which indicates that COC might be one causative factor for vulvar pain.

In women with provoked vestibulodynia the DNIC response to cold noxious stimulation is present in an equivalent way as in healthy women irrespective of use of combined oral contraceptives. The implication of this finding is a better understanding of the endogenous pain inhibitory modulation in vestibulodynia patients. The use of combined oral contraceptives did not affect the DNIC response in healthy women.

In women on combined oral contraceptives the morphology of the vulvar vestibular mucosa displays shallower and sparser dermal papillae compared with that of women in the follicular phase. A similar picture of an increased epithelial volume is seen in women during the luteal phase. The results support the hypothesis of a gestagenic effect on the mucosa.

A higher amount of ER β is expressed in the vulvar vestibular stroma in women on combined oral contraceptives compared with non users. There is a lower concentration of PRB in the stromal tissue of the vulvar vestibule in the luteal phase than in the follicular phase. The results indicate an influence on the steroid receptor expression by ethinyl estradiol as well as by progestins.

There is an increased expression of ER α in the vestibular mucosa in women with provoked vestibulodynia but the epithelial morphology seems unaffected.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

P-pillers effekt på slemhinnan kring slidöppningen samt smärtmekanismer hos friska kvinnor och hos kvinnor med vestibulit.

8.1 BAKGRUND

Samlagssmärta har på senare år blivit en vanlig orsak till gynekologiska öppenvårdsbesök. Förekomsten är osäker men i en svensk studie uppgav ca 13 % av kvinnor i åldern 20-29 år att de haft en längre period av ytlig samlagssmärta. Majoriteten led av vulvavestibulit vilket kännetecknas av långvarig penetrationsmärta samt tryckömheter i området kring slidöppningen; vestibulum. Smärtan medför att de drabbade kvinnorna inte kan ha ett normalt sexuellt samliv, vilket får stora konsekvenser för deras allmänna välbefinnande och partnerrelation.

Orsakerna till vestibulit är inte helt klarlagda. Troligen är det en kombination av flera negativa faktorer, både kroppsliga och psykiska, som samverkar i uppkomsten av smärttillståndet. I sin sjukhistoria uppger patienterna ofta smärta från andra delar av kroppen såsom muskelvärk, huvudvärk samt problem från mag-tarmkanalen. Det är visat att kvinnorna har ett ökat antal ytliga nervfibrer i vävnaden vilka har sänkta smärtrösklar för flera olika stimuli. Kvinnor med vestibulit har även sänkta smärtrösklar på andra delar av kroppen vilket talar för att vestibulit är en del av en mer generaliserad smärtproblematik.

En annan omdiskuterad faktor är ifall p-piller har en negativ effekt på slemhinnan. En klinisk observation antyder att en del kvinnor med ytlig samlagssmärta blir förbättrade enbart genom att sluta med p-piller. Ett par studier har visat ett samband mellan långvarig p-pilleranvändning och vestibulit. Det saknas däremot grundläggande studier som beskriver hur den normala slemhinnan påverkas under menscykeln och under p-pilleranvändning.

Utbudet av effektiva behandlingsmetoder är litet. En kombination av lokal smärtbehandling med bedövningsmedel, bäckenbottenavslappande övningar samt kognitiv beteendeterapi används oftast och är mycket resurskrävande. För att kunna hitta mer effektiva behandlingar måste utlösande orsaker till vestibulit studeras ytterligare.

8.2 VETENSKAPLIGA FRÅGESTÄLLNINGAR

- Har friska kvinnor med p-piller förändrade smärtrösklar i vestibulum jämfört med friska kvinnor utan p-piller? (Delarbete I)
- Har kvinnor med vestibulit en förändrad upplevelse av smärtrösklar generellt, beroende på en försämring av kroppsegna smärthämmande mekanismer? (Delarbete II)
- Finns det en variation av vestibulumslemhinnans uppbyggnad hos friska kvinnor utan p-piller under menscykeln? Skiljer sig vestibulumslemhinnans utseende mellan friska kvinnor med och utan p-piller? (Delarbete III)
- Finns det en variation i vestibulumslemhinnans hormonreceptorhalt hos friska kvinnor under menscykeln? Har friska kvinnor med p-piller förändrad mängd hormonreceptorer i vestibulumslemhinnan jämfört med friska kvinnor utan p-piller? (Delarbete IV)
- Har kvinnor med vestibulit en förändrad mängd hormonreceptorer och uppbyggnad av vestibulumslemhinnan jämfört med friska kvinnor utan p-piller? (Delarbete V)

8.3 MATERIAL OCH METODER

I studierna har sammanlagt ingått 34 kvinnor med vestibulit och 60 friska kvinnor med p-piller samt 64 friska kvinnor utan p-piller.

Smärtrösklar i vestibulumslemhinnan har mätts i det första delarbetet. I delarbete II har vi mätt trycksmärta på ben och arm före och under köldprovokation samt mätt ångest- och depressionsförekomst med två enkäter.

Vävnadsprover har tagits från slemhinnan kring slidöppningen 1-2 gånger i menscykeln. Hormonreceptorer ($ER\alpha$, $ER\beta$, PRA, PRB, AR, GR samt proliferationsfaktorn Ki67) har analyserats med hjälp av immunohistokemi. Slemhinnans utseende och tjocklek har studerats med en nyligen utarbetad datoriserad metod.

8.4 RESULTAT

- Friska kvinnor med p-piller har sänkta mekaniska smärtrösklar i vestibulumslemhinnan jämfört med friska kvinnor utan p-piller (delarbete I).
- Kvinnor med vestibulit har sänkta smärtrösklar på ben och arm men de uppvisar kroppsegen smärtlindring jämförbart med friska kvinnor både före och efter köldprovokation. Kvinnor med vestibulit uppvisar mer ångest och depression än friska kvinnor med och utan p-piller. Friska kvinnor med p-piller skiljer sig ej avseende smärtrösklar på ben och arm jämfört med friska kvinnor utan p-piller (delarbete II).
- Slemhinnetjockleken i vestibulum varierar under menscykeln med längre avstånd mellan förankrande underhudsutskott hos friska kvinnor utan p-piller. Friska kvinnor med p-piller uppvisar både kortare samt glesare förankrande underhudsutskott i slemhinnan. Mängden ytliga blodkärl är ökad hos friska kvinnor med p-piller jämfört med friska kvinnor (delarbete III).
- Det finns färre progesteronreceptorer B(PRB) i vestibulumslemhinnan hos friska kvinnor i senare delen av menscykeln. Kvinnor med p-piller har större mängd östrogenreceptor beta ($ER\beta$) i vestibulumslemhinnan än friska kvinnor utan p-piller (delarbete IV).
- Kvinnor med vestibulit har ökad mängd östrogenreceptor alfa ($ER\alpha$) i vestibulumslemhinnan jämfört med friska kvinnor utan p-piller. Epiteltjockleken i vestibulum skiljer sig ej jämfört med friska kvinnor utan p-piller (delarbete V).

8.5 SLUTSATS

Friska kvinnor med p-piller uppvisar sänkta smärtrösklar i området kring slidöppningen, ökat antal östrogenreceptorer beta ($ER\beta$), ökat antal ytliga blodkärl samt förändrad slemhinneprofil jämfört med friska kvinnor utan p-piller. Dessa fynd indikerar att det kan finnas ett samband mellan p-piller och samlagssmärta.

Under menscykeln hos friska kvinnor sker en variation av både slemhinneprofilen samt progesteronreceptorer B.

Kvinnor med vestibulit har generellt sänkta smärtrösklar på kroppen men uppvisar kroppsegen smärtlindring. Ångest och depression var mer förekommande hos kvinnor med vestibulit. Slemhinnan hos kvinnor med vestibulit innehåller mer östrogenreceptorer alfa ($ER\alpha$) jämfört med friska kvinnor utan p-piller.

9 APPENDIX

9.1 QUESTIONNAIRE

Date:

Patient, No.....

Control, No.....

Age.....

Height.....

Weight.....

- Occupation.....
- Do you smoke?
If yes: How many/day?.....
- Regular sports activities?
If yes: Type of activity?.....
Times/week.....
- Swimming?
- Horse riding?
- Bicycling?

PREVIOUS AND PRESENT DISEASES

- | | | | Year |
|--|----|----------|-------|
| • Have you been treated with antibiotics for : | | | |
| Tonsillitis? | No | Yes | |
| Sinusitis? | No | Yes | |
| Acne? | No | Yes | |
| Urinary tract infection | No | Yes | |
| Other infections? | No | Yes | |
| • Have you had surgery? | No | Yes | |
| If yes: Type of surgery?..... | | | |
| • Regular medications? | No | Yes | |
| If yes: Name of medicine?..... | | | |
| • Do you use any cortisone? | No | Yes | |
| If yes: Indication?..... | | | |
| If yes: Name of medicine?..... | | | |
| • Health food products/nature-cure medicine? | No | Yes | |
| If yes: Name of products/medicine?..... | | | |
| <u>Present or previous:</u> | | | |
| Headache (tension)? | No | Previous | Now |
| Migraine? | No | Previous | Now |
| If yes: Migraine treatment? | No | Previous | Now |
| If yes: Name of medicine?..... | | | |
| Muscle pain? | No | Previous | Now |
| Gastritis? | No | Previous | Now |
| Irritable bowel? | No | Previous | Now |
| Constipation? | No | Previous | Now |
| Urinary urgency without infection? | No | Previous | Now |
| Back pain? | No | Previous | Now |
| Dermatological disease? | No | Previous | Now |
| If yes: What diagnosis?..... | | | |
| If current treatment? What medication?..... | | | |

ALLERGIES

Do you have:		Previous	Now	Treatment
Conjunctivitis?	No	Previous	Now
Rhinitis?	No	Previous	Now
Eczema?	No	Previous	Now
Asthma?	No	Previous	Now
Type of asthma:				
Seasonal?	No	Previous	Now	
Physical strain?	No	Previous	Now	
Infection?	No	Previous	Now	
Other?				
Food allergy?	No	Previous	Now	
If yes: What food?.....				
What symptoms do you get?.....				
Allergy to medication?	No	Previous	Now	
If yes: What medication?.....				
What symptoms do you get?.....				

GYNAECOLOGY

Menstruation

Age at first period?.....			
Regular periods?	No	Yes	
Days in the cycle?.....			
Days of bleeding?.....			
Dysmenorrhoea?	No	Yes	
If yes: Analgesic medication?	No	Yes	
If yes: Name of medication.....			
Date of last period?.....			

Pregnancies

Have you:	No	Yes	Number.....
Been pregnant?	No	Yes	Number.....
Given birth?	No	Yes	Number.....
Miscarriage?	No	Yes	Number.....
Legal abortion?	No	Yes	Number.....
Age at first pregnancy?.....			
Complication:	No	Yes	
During pregnancy?	No	Yes	
If yes: What complication?.....			
At delivery?	No	Yes	
If yes: What complication?.....			
Previous episiotomy?	No	Yes	
If yes: Any complication afterwards?	No	Yes	
What complication?.....			

Birth control

Have you ever used oral contraceptives?	No	Yes
If yes: Age when you first started?.....		
Ever made a pause?	No	Yes
Number of pauses?.....		

Reason for pauses?.....		
Name of pills?.....		
• Current use of oral contraceptives?	No	Yes
If yes: Name of pills?.....		
• Total time of oral contraceptives?.....		
• Side effects of oral contraceptives?	No	Yes
If yes: What side effect?.....		
• Indication for oral contraceptives:		
Dysmenorrhoea?	No	Yes
Birth control?	No	Yes
Before your first intercourse?	No	Yes
Irregular periods?	No	Yes
• Do you use:		
No birth control?	No	Yes
Condom?	No	Yes
Pessary?	No	Yes
Intrauterine device?	No	Yes
If IUD:		
Cooper?	No	Yes
Hormonal?	No	Yes

PREVIOUS GYNAECOLOGICAL INFECTIONS

• Chlamydia?	No	Yes
If yes: Salpingitis?	No	Yes
• Gonorrhoea?	No	Yes
• Trichomonas?	No	Yes
• Genital herpes?	No	Yes
If yes: Year of first infection?.....	No	Yes
Localisation of blisters?.....	No	Yes
How often blisters?.....	No	Yes
• Condyloma	No	Yes
If yes: Localisation of warts?.....		
Flat condyloma?	No	Yes
Previous treatment?	Podophyllotoxin	Laser?
• Often foul smelling discharge?	No	Diathermy?
If yes: Treatment	Tablets	Yes
• Previous yeast infection? Never	2 times	Vaginal cream
If yes: Age at first infection?.....	3-9 times	Nothing
If several infections: How often?	≥ 10 times	
Usually local treatment, topical/ vaginal?	Every month	< 3/year
Previous oral treatment, fluconazol/itraconazol?	No	Occasionally
If yes: Single dose?	No	Yes
1 dose/day?		
1 dose/week?		
1 dose/month		
Time of treatment?.....		
• Is the treatment currently effective?	No	Yes
• Was the treatment previously effective?	No	Yes
• Do you eat sweets? Never	Occasionally	Moderately
		Frequently

HYGIENE

- Do you use in the genital area? Soap Only water Oil
- Tampon? Never Occasionally Most often
- Sanitary napkins? Never Occasionally Most often
- Do you usually wear tight panties? Never Occasionally Most often
- If you have pain during intercourse and/or using tampons:
 - When did the pain start?.....
 - Did you use oral contraceptives when the pain started? No Yes
 - Did you use oral contraceptives before the pain started? No Yes
 - Is there a connection to a special event when the pain started? No Yes
 - If yes: What connection?.....
- Around the vaginal opening, do you have:
 - Itch? No Previously Now
 - Dryness? No Previously Now
 - Fissures? No Previously Now
 - Painful urination? No Previously Now
 - Painful ovulation? No Previously Now
 - Pain at sexual arousal? No Previously Now
 - Pain wearing tight panties? No Previously Now
- Is the pain:
 - Continuous? No Previously Yes
 - In periods? No Previously Yes
- Is the pain related to the menstrual period? No Yes
- If you have pain during intercourse, is it most painful:
 - During penetration? No Yes
 - During the whole intercourse? No Yes
 - Afterwards? No Yes
 - If yes: For how long time?..... No Yes
- Have you ever got an explanation of your pain?
 - If yes: What explanation/diagnosis?.....

SEXUALITY

- Age at first intercourse?.....
- Number of partners? Total..... Previous year.....
- Current partnership? No Yes
- Length of partnership?..... No Yes
- Did you have a good sexual relationship with your partner before your pain started? No Yes
- Did you previously have a good sexual relationship No Yes
- How often did you have intercourse before your pain started?
 - Every day?
 - Several times/week?
 - Every week?
 - Occasionally?
- How often do you have intercourse now?.....
- Does your partner want to have sex more often? No Yes
- Has your partner always wanted to have more sex than you? No Yes
- Does the pain influence your relationship? No Yes

• Does your partner understand your problem?	No	Yes
• Did any previous relationship break off due to your pain?	No	Yes
• Do you currently feel desire for intercourse?	No	Yes
• Did you previously feel desire for intercourse?	No	Yes
• Does masturbation work better than intercourse?	No	Yes
• Do you have orgasm?	No	Yes
• Could you previously have orgasm?	No	Yes
• Do you feel dry in the vagina?	No	Yes
• Did you previously feel dry in the vagina?		
• Have you ever experienced sexual threat or violence?		
PARTNER		
• Did he previously have:		
Condyloma?	No	Yes
Herpes?	No	Yes
Prostata infection?	No	Yes
Yeast infection?	No	Yes

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.....

.....

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