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STUDIES ON THE EXTRACELLULAR MATRIX OF THE DYSFUNCTIONAL PELVIC FLOOR

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Abstract

Objective: Pelvic floor dysfunction cause impaired quality of life of many women and is one of the most common reasons for gynaecological surgery on benign indications. Little is known about the pelvic floor extracellular matrix as a pre-requisite for mechanical sustainability through vaginal delivery, age and hormonal changes. For this reason, pelvic floor extracellular matrix was investigated in women suffering from the two major dysfunctional conditions, pelvic organ prolapse (POP) and stress urinary incontinence (SUI), regarding molecules responsible for tensile strength and elasticity.
Methods: Para-urethral biopsies were collected from women with POP, SUI and compared to healthy controls matched according to menopausal status or age. The collagen concentration and extractability by pepsin digestion were analyzed in women with POP. Real-time RT-PCR revealed the gene signals and immunohistochemistry the protein expressions of collagen I, collagen III, the small leucin-rich proteoglycans (SLRPs) decorin, lumican and fibromodulin as well as the elastin associated proteins fibrillin-1 and fibulin-5 in POP and SUI. In SUI the sex steroid hormone receptor isoforms and subtypes ER-α, ER-β, PR-(A+B), PR-B and AR were identified and quantified by scoring and image analysis (ER-α and ER-β) completed by mRNA expressions analyze of ER-α, ER-β, PR and AR by real-time RT-PCR.
Results: The collagen concentration was 30 % lower in the pelvic floor ECM of women younger than 53 years suffering from POP. There were lower mRNA expressions of all the investigated SLRPs and the elastin associated fibulin-5 in pre-menopausal women with POP. A 16-fold reduction of decorin mRNA was most prominent with a corresponding weaker protein expression of decorin. Postmenopausal women with POP exposed significantly lower mRNA expressions of fibromodulin and fibulin-5. A significant reduction of the mRNA expression of fibrillin-1 seen in women with SUI irrespective of menopausal status was confirmed by a lower immunoreactivity. All hormone receptor isoforms or subtypes were expressed in the pelvic floor ECM with ER-β showing an increased expression in pre-menopausal women with SUI. A corresponding elevation in gene expression was not discovered.
Conclusion: Evidence for POP and SUI deriving from different alterations in the pelvic floor ECM has been found. The results therefore suggest different pathophysiological backgrounds to these conditions on the tissue level. Furthermore are the greatest changes found in pre-menopausal POP, reflecting the severity of pelvic floor dysfunction in this group. All investigated hormone receptor isoforms or subtypes were expressed. The ER-β protein was more expressed by in pre-menopausal women with SUI.

Key words: *Pelvic floor, pelvic organ prolapse, stress urinary incontinence, extracellular matrix, collagen, SLRPs, decorin, lumican, fibromodulin, elastin associated proteins, fibrillin-1, fibulin-5, sex steroid hormone receptors, ER, PR, AR*

To Sofia, John and Olle

List of Publications

I. Söderberg MW, Falconer C, Byström B, Malmström A, Ekman G
Young women with genital prolapse have a low collagen concentration
Acta Obstet et Gynecol Scand 2004: 83: 1193-1198

II. Söderberg MW, Byström B, Kalamajski S, Malmström A, Ekman-Ordeberg G
Down-regulations of small leucine–rich repeat proteoglycans and fibulin-5 in pelvic organ prolapse
Submitted

III. Söderberg MW, Byström B, Hammarström M, Malmström A, Ekman-Ordeberg G
Decreased expression of fibrillin-1 in stress urinary incontinence
Submitted

IV. Söderberg MW, Johansson B, Masironi B, Byström B, Falconer C, Sahlin L, Ekman Ordeberg G
Pelvic floor sex steroid hormone receptors, distribution and expression in pre- and postmenopausal stress urinary incontinent women
Acta Obstet and Gynecol Scand 2007: 86: 1377-1384

Contents

List of abbreviations

AR	Androgen receptor
cDNA	Complementary DNA
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EMILIN	Elastin microfibril interface located protein
ER	Estrogen receptor
GAG	Glycosaminoglycan
HRT	Hormone replacement therapy
LOX	Lysyl oxidase
LOXL	Lysyl oxidase like
LTBP1	Latent TGFβ binding protein1
MAGP	Microfibril associated glycoprotein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NR	Nuclear receptor
POP	Pelvic organ prolapse
POP-Q	Pelvic organ prolapse quantification system
PR	Progesteron receptor
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SHBG	Steroid hormone binding protein
SLRP	Small leucine-rich repeat proteoglycan
SNRI	Serotonin norepinephrine reuptake inhibitor
SUI	Stress urinary incontinence
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of MMP
TVT	Tension-free vaginal tape

Background

INTRODUCTION

The female pelvic floor

The pelvic floor is situated at the bottom of the abdominal cavity and closes the canal within the bony pelvis (DeLancey, 1993). It consists of a layer of interconnecting striated muscles, the levator ani muscles, containing openings allowing passage from the urethra, the vagina and the anal canal. There is a static contraction in these muscles that can be increased voluntarily. Connective tissue is inserted between the striated muscle cells, surrounding the muscles and organs as fascias and forming ligaments for suspension. There is a confluence of this fibrous connective tissue laterally inside the muscle layer named arcus tendineus fascia pelvis or "the white line", fig1. It emanates from the ischial spine and passes down-wards parallel to the vagina. The general designation of all connective tissue in the pelvic floor is the endopelvic fascia (DeLancey, 1993).

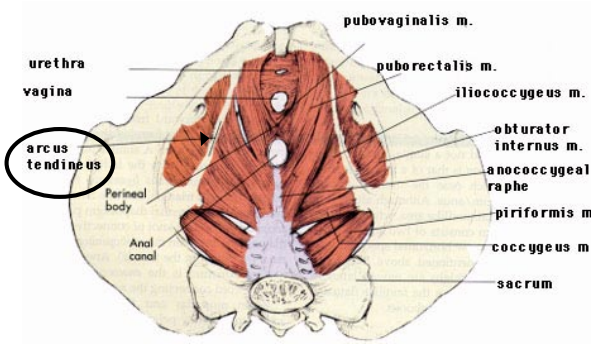


Fig 1. The pelvic floor, inside view.

Pelvic floor dysfunction

Pelvic floor dysfunction negatively affects the quality of life negatively of many women. It is not life threatening, but the constant reminders of the shameful symptoms are not only restraining the sufferer from activities and cause a deterioration in sex life but also diminishing the self-esteem (Barber *et al.*, 2002; Rortveit *et al.*, 2007; Handa *et al.*, 2008). An additional sense of guilt for having failed during childbirth, lifting too heavy or not exercising the pelvic floor enough prevents these, often otherwise healthy and capable women from seeking medical care. To help all these women by clarifying predisposing circumstances, we have studied the two major conditions at the tissue level: pelvic organ prolapse (POP) and stress urinary incontinence (SUI).

PELVIC ORGAN PROLAPSE

Definition

POP-Q is the standard system of terminology for description of POP approved by the International Continence Society (Bump *et al.*, 1996). It defines POP as descent of one or more of: the anterior vaginal wall, the posterior vaginal wall and the apex of the vagina or vault after hysterectomy, fig 2. A system of stages describes the extent of the descent and stage ≥ II being considered pathological after vaginal childbirth. The descent should be evaluated as maximal bulging during straining by the patient or traction by the examiner.

Table 1 Staging of POP according to the POP-Q system

Stage 0:	No prolapse
Stage I:	The most distal portion of the prolapse is > 1 cm above the hymen level
Stage II:	The most distal portion of the prolapse is ≤ 1cm proximal or distal to the hymen level
Stage III:	The most distal portion of the prolapse is > 1cm below the hymen level, but protrudes no further than 2 cm less than the total vaginal length
Stage IV:	Complete eversion (less than 2 cm of the total vaginal length is not protruded)

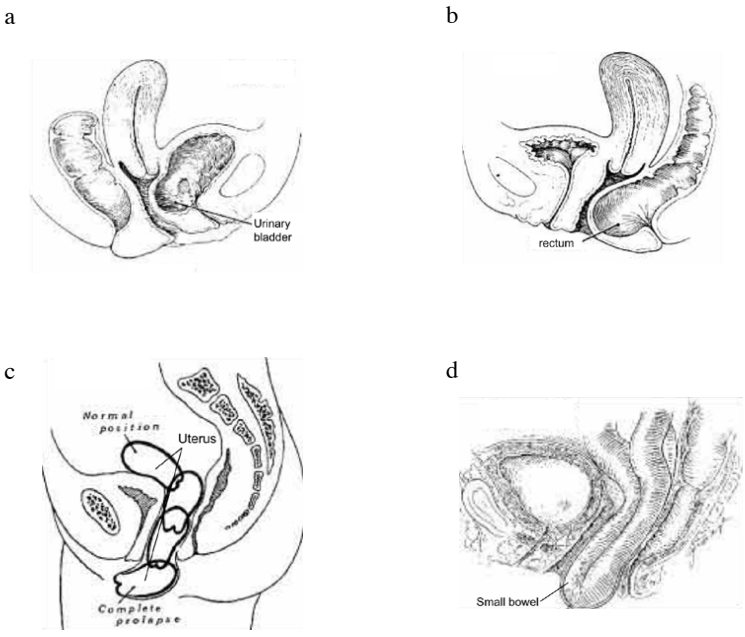


Fig 2. POP in a. anterior compartment b. posterior compartment c. middle compartment, non-hysterectomized d. middle compartment, hysterectomized

Prevalence and predisposing clinical factors

POP is a common reason for gynaecological surgery after menopause and the lifetime risk of surgery for POP or SUI is estimated to 11 % in a U.S. population (Fialkow *et al.*, 2008). However, there are reasons to believe that many women lack adequate treatment. In a Swedish questionnaire study of POP specific symptoms in a large population, the prevalence was 8.3 % in women of 30-79 years of age (Tegerstedt *et al.*, 2005). It increased with age up to 60-69 years and declined slowly thereafter, substantiating the fact that POP affects women at an age when women today are still active.

Many studies have been conducted on factors increasing the risk for POP. The main factor that predominates in every study is vaginal delivery increasing with subsequent deliveries. Other factors with a positive correlation are weight of largest infant, obesity and age (Mant *et al.*, 1997; Olsen *et al.*, 1997; Samuelsson *et al.*, 1999; 2000; Swift *et al.*, 2001; Tegerstedt *et al.*, 2006; Rortveit *et al.*, 2007).

There are indications of a hereditary component in development of POP, not least in every day clinical practice, where many patients recognize their condition in mothers and sisters. Scientifically, this has not been proved convincingly, but a few studies are pointing in this direction (Chiaffarino *et al.*, 1999; Rinne *et al.*, 1999). However, there seems to be correlations, particularly in younger women, to joint hyper-mobility, rheumatologic and neurological diseases and even Ehlers' Dunlos syndrome (McIntosh *et al.*, 1995; Norton *et al.*, 1995; Strohbehn *et al.*, 1997; Bai *et al.*, 2002). Furthermore, racial differences have been described, with POP less common in Afro-Americans compared to that in US Caucasians (Rortveit *et al.*, 2007). Another indirect sign may be, that risk factors for POP recurring after surgery are not only a high grade POP from the anterior vaginal wall, but also POP developing in younger years, suggesting a perhaps inherited vulnerability in these women (Whiteside *et al.*, 2004; Diez-Itza *et al.*, 2007; Jeon *et al.*, 2008; Miedel *et al.*, 2008)

Patophysiology

Current opinion on POP development suggests a combination of lack of underlying support from the levator ani muscles combined with stretch of the endopelvic fascia, leading to tissue weakening and thus herniation from an adjacent organ into the vagina (DeLancey, 1993). The insufficiency of muscular function is considered being due to mechanical damage of muscles and/or nerves (Smith *et al.*, 1989; DeLancey *et al.*, 2003). In the past ten years researchers have sought additional explanation of why the pelvic floor connective tissue in some women is less resilient to the trauma of vaginal birth. Different ECM components have been studied, but the results are contradictory and hard to interpret since different techniques have been used and the biopsy sites differ and are more or less affected by the stretch of the prolapse (Jackson *et al.*, 1996; Ewies *et al.*, 2003; Wong *et al.*, 2003; Moalli *et al.*, 2005; Song *et al.*, 2007; Edwall *et al.*, 2008; Klutke *et al.*, 2008). It is also important to differentiate between SUI and POP in the study groups and to match thoroughly for age.

Symptoms and diagnosis

The only symptom that can be clearly associated to POP is bulging, i.e. the sensation of something coming out of the vagina (Tegerstedt *et al.*, 2005). Other symptoms, more related to the site of the prolapse, may be bladder emptying difficulties and /or urge in anterior wall POP or defaecatory problems in posterior wall POP. Sensations as heaviness or pressure are considered unspecific and cannot directly be related to occurrence of POP.

A thorough gynaecological examination is necessary for diagnosing POP adequately. It should include a description of protrusion of the relevant sites: the anterior compartment (anterior vaginal wall), the posterior compartment (posterior vaginal wall) and the middle compartment (cervix / uterus or vaginal vault after hysterectomy). An additional description of the perineum (skin and underlying perineal

body) is optimal. This is the preferred way to describe POP rather than the nomenclature based on the organ behind the bulgeing, as this is difficult to finally determine before surgery. The fully detailed POP-Q system can be used for staging, but in clinical practice it is more convenient to describe the compartment from which the protrusion(s) originate(s), its relation to the hymen level and hence the stage (0-IV).

Treatment

The consensus today is that treatment of POP should be based on the inconvenience of the POP related symptoms rather than the anatomical defect found at the examination. As a conservative alternative, a vaginal pessary can be considered for symptom relief. The efficacy and tolerance is individual. The user needs regular medical check-ups and the sexual activity can be affected negatively. The pessary does not prevent the prolapse from extending and side effects as vaginal discharge and ulcers can occur early or later in the treatment period.

Surgical treatment of POP is documented as early as 1521, in the form of a vaginal hysterectomy (Emge *et al.*, 1966). The Manchester procedure was introduced in 1888 (Donald, 1903). It consists of anterior and posterior repair combined with cervical amputation. The posterior repair is extended with midline sutures of the levator ani muscles and prolongation of the perineum leaving the vaginal intoitus very narrow. Modified forms are still employed today. They focus on the compartment responsible for symptoms, since there is no convincing evidence that preventive surgery in a non-symptomatic compartment is of use. It is also considered of utmost importance to avoid post-operative dyspareunia, preserve bladder and bowel function and thus focus on the functional result rather than the anatomical. This treatment shift has led to levator suturing and other techniques resulting in an exaggerated closure of the vagina or other mutilating procedures are uncommon. Another approach to POP in the middle compartment, apart from cervical amputation or vaginal hysterectomy, is attaching the vagina to an inert part of the pelvis either by vaginal fixation to the sacro-spinousos ligament or abdominal sacrocolpopexy either by laparoscopy or laparotomy using different foreign material fixation techniques.

POP recurrence after surgery is however not uncommon with anatomical recurrence rates up to 40 % at examination 5 years after surgery (Miedel *et al.*, 2008). Investigations concerning POP recurrence are generally hard to interpret due to lack of information as to whether the recurrent site is identical to the operated site. Furthermore most studies deal with re-operation rates without reporting POP related symptoms (Clark *et al.*, 2003). According to a recent Swedish study 20 % had recurrent POP symptoms 5 years after primary surgery and 10 % had undergone a second operation (Miedel *et al.*, 2008) . Recurrent POP is, moreover, more common in the anterior compartment, when the primary prolapse is of a high stage and in a younger population. This information, combined with the fact that the risk of an additional recurrence increases after the first re-operation, has raised the question whether the vaginal supporting tissue is too weak in these women and needs to be augmented by foreign material (Clark *et al.*, 2003).

For this reason the medical industry, inspired by the success in hernia surgery, has in the last ten years introduced implants for vaginal repair. Both biological material, such as collagen produced from pig skin or pig bowels, and synthetic material, such as prolene normally used in the TVT® tape, has been introduced in different shapes to be positioned and fixated with different techniques between the protruding vaginal wall and the adjacent organ. These procedures are currently being evaluated and one of the reported problems being erosion of the vaginal wall (Pacquee *et al.*, 2008).

Incorporation of foreign material in these particularly delicate and probably primarily impaired vaginal tissues in women suffering from POP fully justifies the need for basic research on the tissue level.

STRESS URINARY INCONTINENCE

Definition

According to the International Continence Society SUI is defined as: the complaint of involuntary leakage on effort or exertion, or on sneezing or coughing (Abrams *et al.*, 2002).
Urinary incontinence as a whole is defined as: the complaint of involuntary leakage. The other common forms of incontinence are urge incontinence and mixed incontinence. Urge incontinence is defined as: the complaint of involuntary leakage accompanied by or preceeded by urgency (urge to micturate) and mixed incontinence is defined as: the complaint of involuntary leakage associated with urgency and also on effort or exertion, or on sneezing or coughing.

Prevalens and pre-disposing clinical factors

According to Swedish epidemiological studies based on questionnaires, the prevalence of leakage varies from 10-70 % in 60-70 year old women and 8-65% in 40-50 year old women (Tegerstedt *et al.*, 2005). The lower figures represent frequent and the higher occasional loss of urine.
In all studies evaluating risk factors for SUI, vaginal delivery turns out to be the single most linked factor with additive effects of multiparity and leakage after the first delivery (Goldberg *et al.*, 2005; Altman *et al.*, 2006; Ekstrom *et al.*, 2008). A high body mass index and previous hysterectomy has also been shown to increase the relative risk (Goldberg *et al.*, 2005; Altman *et al.*, 2007).
Hormonal replacement therapy (HRT) to postmenopausals, either estrogens orally with or without progestin supplement or locally administered estrogens, has often been used in clinical practice as a part of conservative treatment of SUI. Few studies have confirmed a beneficial effect and the large Womens' Health Initiative Study reported a negative effect of HRT on SUI (Ishiko *et al.*, 2001; Robinson *et al.*, 2003; Hendrix *et al.*, 2005).
As in POP, there is a clinical impression that SUI "runs in the family". A genetic association has been confirmed by a questionnaire study concerning close relatives and twin studies (Ertunc *et al.*, 2004; Rohr *et al.*, 2004; Altman *et al.*, 2008). This reinforces the theory that a constitutional vulnerability increases the risk for SUI after childbearing.

Patophysiology

Two theories dominate concerning SUI development, the urethral hypermobility theory and the intrinsic urethral sphincter theory. The first applies in this context to younger women, the hypermobility of the anterior vaginal wall being looked upon as overextended tissue due to loss of resilience. The second theory concerns mostly elderly women with stiff tissues.

The urethral hypermobility theory
The urethral hypermobility theory, also named the integral theory, states that pelvic floor integrity is a result of balancing forces (Ulmsten, 1997). The optimal location of the bladder neck for closure is maintained with increasing abdominal pressure in a healthy woman, but an imbalance leads to a hyper-mobile anterior vaginal wall dislocating the bladder neck and leading to SUI, fig 3.

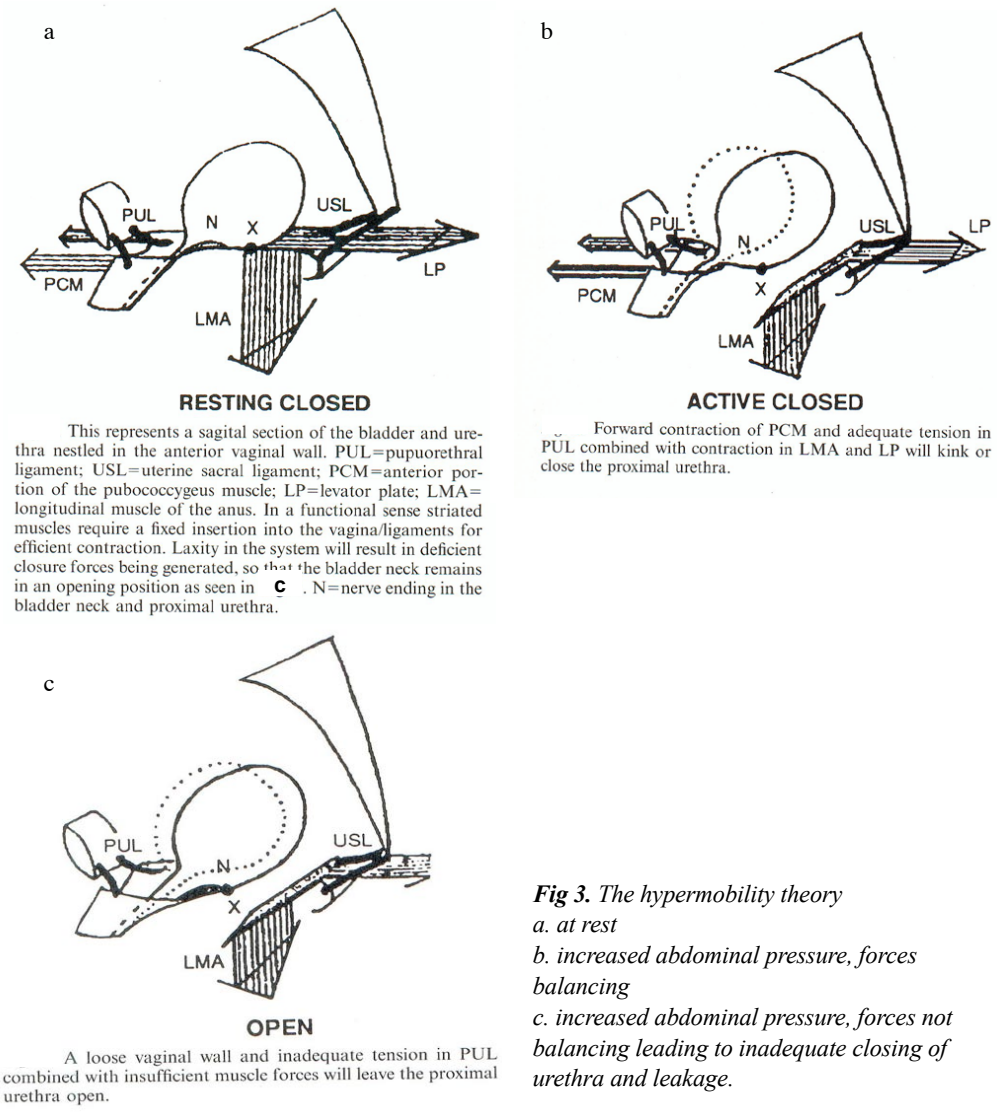


Fig 3. *The hypermobility theory*
a. at rest
b. increased abdominal pressure, forces balancing
c. increased abdominal pressure, forces not balancing leading to inadequate closing of urethra and leakage.

Intrinsic sphincter deficiency theory

Magnetic resonance studies have revealed that there exists an exterior striated and inner smooth muscular sphincter of the female urethra (Macura *et al.*, 2007). A thinner and shorter internal muscular layer and funnelling of the bladder neck has been associated to SUI, particularly when reflected by a low urethral pressure and not necessarily with concomitant hypermobility, fig 4.

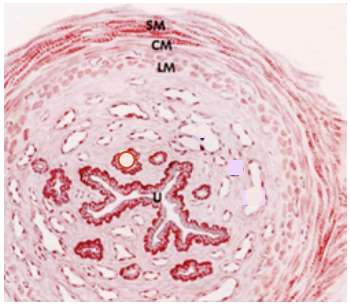


Fig 4. *Female urethra, cross-sectionat the level of the sphincter. SM= striated muscular layer connected with pelvic floor muscles. CM= circular smooth muscle LM= longitudinal smooth muscle U= urethral lumen*

Symptoms and diagnosis

SUI is a symptomatic condition and the sense of severity of symptoms is very individual. The key questions to ask at the consultation or in a questionnaire are whether leakage occurs during increased abdominal pressure such as physical activity like jumping and running or coughing or sneezing. A micturition diary can confirm the leakage and assure normal voiding habits. At a gynaecological examination the hypermobility of the anterior vaginal wall and leakage during coughing can be visualized. The bladder can be filled with sterile water and the patient can be asked to stand or even jump to provoke the leakage.

In cases of difficulty to separate urinary incontinence conditions the patient can undergo a urodynamic examination (Lose, 1997). The intravesical and intra-urethral pressures are measured while the urinary bladder is being filled and during coughing. Negative urethral pressure during cough provocation prompts a diagnosis of SUI, and this is often confirmed by leakage during this phase of the examination. A pad-test, i.e. weighing the protection pad before and after usage, can be used to estimate the amount and frequency of leakage. It is however disputed how far the amount or frequency of the leakage should influence the treatment decision, since for example a marathon runner may have higher demands on her body function as someone less physically active.

Treatment

The conservative treatment of SUI is pelvic floor exercise to strengthen the pelvic floor muscles, both in voluntary contractions to elevate the pelvic floor when needed, but also to increase the resting tonus. Studies have shown improvement in particular in cases of smaller leakages (Hay-Smith *et al.*, 2001; Burgio *et al.*, 2003).

Over the years many surgical methods have been tried to stabilize the bladder neck in order to cure SUI. Success rates have been moderate, recurrences common and complications such as voiding difficulties bothersome. The hypermobility theory claimed by Ulf Ulmsten and Papa Petros led to the insight that a tension-free support under the mid-urethra would be more physiological, and the intravaginal sling procedure followed by the TVT® procedure was introduced in 1993 (Ulmsten *et al.*, 1996). This method, where a mesh of polypropylene tape is loosely inserted under the anterior vaginal wall at mid urethral level with a special device and using local anaesthesia, has revolutionized SUI treatment, fig 5. It is a standardized quick procedure suitable for day-care clinics, with high short- and longer-term cure rates and few complications (Ankardal *et al.*, 2006). To further simplify the operation, the ends of the

tape were drawn through the pelvic obturator membrane instead of behind the symphysis parallel to the bladder, thus avoiding the risk of bladder perforation, fig 6. This obturator method was introduced a few years ago and evaluation so far has shown figures in parity with TVT® (Rinne *et al.*, 2008). Today the TVT® is considered the worldwide gold standard operation for SUI, which probably will be followed in the Nordic countries by the most common obturator method, TVT-O®.

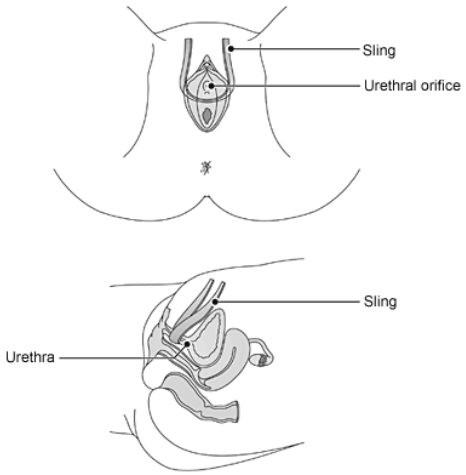


Fig 5. *The TVT- procedure*

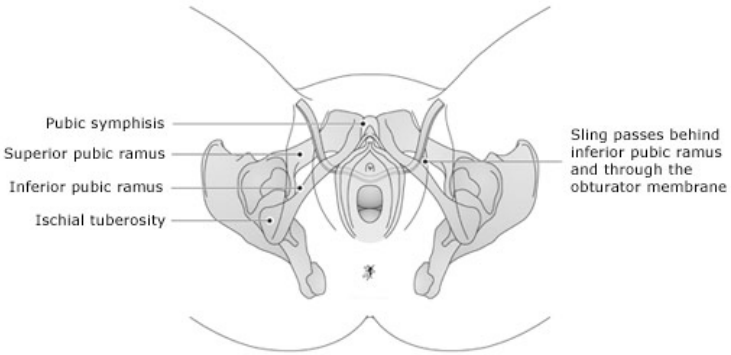


Fig 6. *The TVT procedure with transobturator technique (TVT-O)*

Injection of bulking substances in the urethral wall, is a treatment alternative based on the ISD theory. Different substances have been used and so far the outcomes are moderate in terms of cure rate and complications (Keegan *et al.*, 2007). This is however considered a treatment alternative when TVT® for some reason is not possible.

The resting tonus of the intrinsic urethral sphincter can be reinforced by duloxetin, a SNRI drug discovered during clinical trials for depressive conditions (Jost *et al.*, 2004). The efficacy is moderate, and is reversible when the drug is withdrawn, for which reason its treatment potential is considered limited (Mazo *et al.*, 2004).

FIBROUS CONNECTIVE TISSUE EXTRACELLULAR MATRIX

General contents

The contents of the ECM are produced by the sparsely-distributed fibroblasts or myofibroblasts, fig 7. In addition, there are endothelial cells surrounding vessels and inflammatory cells. The basal membrane delimits the ECM from the epithelial layer.

Collagen I and III are the dominating molecules responsible for tissue strength, and the elastic fibres enable the tissue to stretch (Gelse *et al.*, 2003; Mithieux *et al.*, 2005). Hylaluronan, a glykosaminoglycan (GAG) with viscoelastic properties, is important for the water content of the ECM and for transportation of cells, and is active in the inflammatory response (Wiig *et al.*, 2008).

Proteoglycans consist of a GAG chain and a core protein. They are divided into three main families: the larger hyalectans, the small leucine-rich repeat proteoglycans (SLRPs) and the heparan sulphate proteoglycans (Iozzo, 1998). They appear in different remodelling contexts in the ECM such as fibril organization, mediating cell adhesion, migration, proliferation, differentiation and interaction with growth factors and cytokines.

Other proteins central in the very dynamic ECM are cell-surface adhesive proteins such as intergrins, ECM-interacting proteins such as fibronectins, the inflammatory cytokines, growth factors, the protein-degrading matrix metalloproteinases (MMPs) and the tissue inhibitors of MMPs (TIMPs). The NRs are cellular proteins acting in the nucleus after ligand binding (Beato *et al.*, 2000)

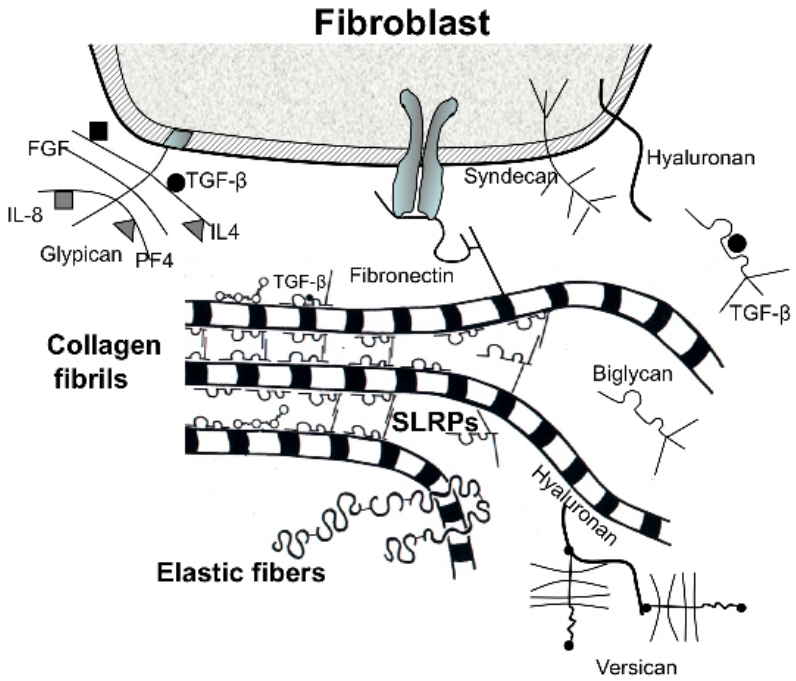


Fig 7. *The extracellular matrix of fibrous connective tissue*

Collagens

The collagen family consists of 29 members so far known.

Table 2 The collagen family listed below by their structural groups:

Structural group	Collagen type
Fibril-forming	I, II, III, V, XI
Basement membrane	IV
Microfibrillar	VI, XXVIII
Anchoring fibrils	VII
Hexagonal network-forming	VIII, X
FACIT (fibril-associated collagens with interrupted triple helices)	IX, XII, XIV, XIX, XX, XXI
Transmembrane	XIII, XVII, XXIII, XXV
Multiplexins	XV, XVI, XVIII
No group	XXII, XXIV, XXVI, XXVII, XXIX

Types I, II, III, V and XI are fibril-forming. Collagen I is the most abundant and best studied (Gelse *et al.*, 2003). It is the major collagen in fibrous connective tissue and bone and is the dominant contributor to tensile strength. Mutations in the gene encoding collagen I are causing osteogenesis imperfecta and one form of Ehlers' Dunlos syndrome (Prockop *et al.*, 1995). Collagen III is co-appearing and interacting with collagen I in fibrous connective tissue but also in interstitial tissues and smooth muscle and has an expanding function. Familial aortic aneurysm and another form of Ehlers' Dunlos syndrome are due to collagen III gene mutations.

Of the other fibrillar collagens, type V has a somewhat similar distribution to collagen III except for its additional appearance in bone. Collagen II and XI are occurring in cartilage and the vitreous body.

Procollagen I is synthesized within the fibroblast where the three chains, two α-1 chains and one α-2 chain, undergo posttranslational modifications, fig 8. Hydroxylations of the frequently occurring amino acids proline and lysine to hydroxyproline and hydroxylysine enable the molecules to form a triple helix with a telopeptide follwed by propeptide at the C- and N-terminal respectively. The procollagens are secreted via the Golgi compartment to the ECM where the propeptides are cleaved off by specific proteinases leaving the tropocollagen molecule with a weight of 300 kD. Thereby the assembly into staggered fibrils with gaps and overlaps starts, involving hydrophobic and electrostatic interactions stabilized by covalent intermolecular cross-links, fig 9. Additional intermediate divalent cross-links are formed by catalyzation of lysyl oxidase between the helical lysine or hydroxylysine residues to those of the telopeptides in an adjacent triple helix. During maturation of the tissue the reducible intermediate divalent cross-links are converted to non-reducible trivalent cross-links involving yet another telopeptide.

Collagen III synthesis has not been studied as detailed as the collagen I synthesis, but is considered similar, with the exception of its triple helix containing three α-1 chains.

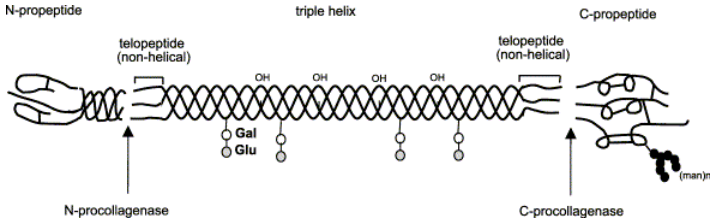


Fig 8. *Procollagen, triple helix*

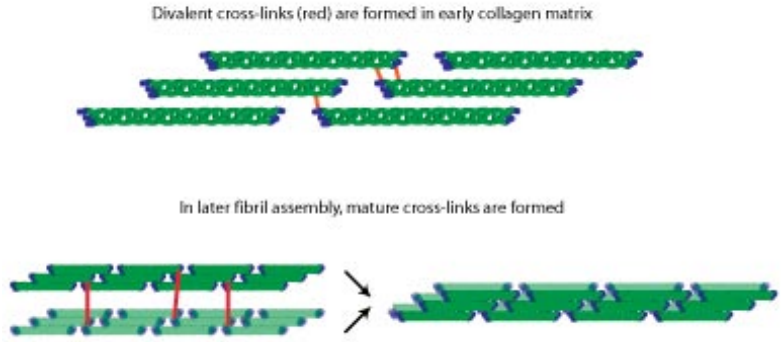


Fig 9. *Collagen cosslinking*

Small leucine-rich repeat proteoglycans (SLRPs)

Fourteen SLRPs have been identified, characterized by tandem repeated leucine –rich domains of 20-30 amino acids each (Matsushima *et al.*, 2000). They are divided into 5 classes from their GAG chains, cysteine rich sequence at the N-terminal ends and number of exons in the genes. Decorin belongs to class I, while fibromodulin and lumican belong to class II.

Decorin

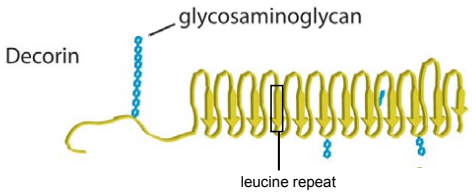


Fig 10 *The decorin molecule.*

Decorin is abundant in connective tissue ECM and has molecular weight of 36 kD, fig 10. Studies have been performed on decorin deficient mice and impaired collagen fibrillogenesis impacting mechanical strength in tendon and skin as well as delayed wound-healing has been demonstrated (Jarvelainen *et al.*, 2006; Zhang *et al.*, 2006). In both studies a compensatory increase of another class I SLRP, biglycan, was noted. There is a presumable role for decorin in collagen cross-linking, but it has not yet been confirmed in vivo. Its binding to collagen is identical to the biglycan binding site near the lysine residues, which are active in the cross-linking process (Kalamajski *et al.*, 2007). Decorin influences the collagen fibril size and the fibrils display a larger diameter in tendons of decorin deficient mice (Danielson *et al.*, 1997; Graham *et al.*, 2000).

Several studies have shown that decorin binds TGF-β which has a potential impact on cell and ECM signalling systems. Initially decorin was discovered being a negative regulator of TGF-β, but subsequent studies have revealed more complex interactions depending on cell type, involving different pathways and compensatory mechanisms (Yamaguchi *et al.*, 1990; Cabello-Verrugio *et al.*, 2007). It is however elucidated that decorin, by inhibiting TGF-β effects, prevents fibrosis in mice muscle wounds (Li *et al.*, 2007; Zhu *et al.*, 2007). Additionally, decorin can bind to and stimulate the synthesis of fibrillin-1, a key-player in the elastic fiber assembly. (Trask *et al.*, 2000; Schaefer *et al.*, 2007).

Fibromodulin and lumican

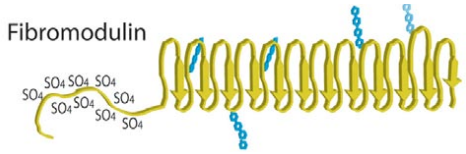


Fig 11 The fibromodulin molecule.

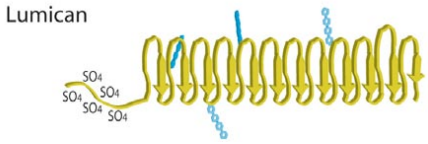


Fig 12 The lumican molecule.

Fibromodulin and lumican are both to class II SLRPs and compete for the same binding site which appears to be near the gap on the collagen fibril (Svensson *et al.*, 2000; Kalamajski *et al.*, 2007). Fibromodulin has a molecular weight of 42 kD and lumican 38 kD, fig10, fig 11. They are important for collagen assembly as fibromodulin deficient mice develop irregular broken tendon fibrils, both larger and thinner than normal (Svensson *et al.*, 1999; Ezura *et al.*, 2000). Seemingly, fibromodulin prevents lateral accretion of the collagen fibril .In fact, evidence for fibromodulin influencing the juxtaposition of collagen fibrils and thereby enabling optimal sites for the intermolecular collagen cross-links, has been found in an unpublished investigation (Kalamajski *et al.*, 2008). Similar effects can be anticipated for other SLRPs, probably influencing their respective specific sites on the collagen fibril. In mice with deleted gene expression of lumican the skin is fragile.In combined fibromodulin /lumican deficiency there are augmented weaknesses in tendons and skin and an additional joint affection resembling Ehlers' Dunlos syndrome (Chakravarti *et al.*, 1998; Ezura *et al.*, 2000; Jepsen *et al.*, 2002).

The Elastic Fibers

The elastic fibers consist of the rubber-like protein elastin mounted on scaffolding microfibrils (Mithieux *et al.*, 2005). A number of additional elastin associated proteins are needed for the elastic fiber assembly, fig 13. Its specific mechanical property is the ability to stretch and recoil.

Elastin

Elastin is produced by myofibroblasts, chondroblasts , endothelial cells and mesothelial cells (Mithieux *et al.*, 2005). Elastogenesis occurs mainly during late foetal and early neonatal periods. Elastin is extremely durable with a half-life estimated to 70 years. In adult life there is a low turn-over unless the elastic fibres are injured, which stimulates neosynthesis. Elastin is synthesized as tropoelastin, encoded from a single gene and stimulated by elastin decay and TGF-β1. Tropoelastin can exist in solution in two forms, an open globar monomer or a distended polypeptide. It is chaperoned by a binding protein when secreted to the ECM by the Golgi complex. It is delivered to the microfibrillar site to which it cross-links and develops from monomer to the polymer elastin. This further cross-linking is performed by members of the lysyl oxidase enzyme family. Desmosine, which is elastin-specific, is one of the cross-link types that can arise.

Elastin-deficient mice die a few days after birth from vascular obstruction due to over-proliferation of smooth muscle cells (Wagenseil *et al.*, 2007). In humans a suppression of the elastin gene is seen as an effect of a chromosomal microdeletion leading to Williams-Beuren syndrome (Ewart *et al.*, 1994; Gilbert-Dussardier, 2006). In this developmental disorder displaying deviant behaviour and face dysmorphism, 75 % suffer from cardiac defects, mainly supravalvular aortic stenosis .
Elastin plays an additional role in ECM remodelling (Mithieux *et al.*, 2005). It can regulate arterial and lung terminal airway branching morphogenesis and through the elastin-laminin receptor for example mediate regulation of skin fibroblast proliferation, myoblast proliferation, chemotaxis for monocytes and fibroblasts and inhibition of the migratory response of myoblasts to chemo-attractants. Another study visualized bindings between α-elastin and SLRPs decorin and biglycane (Itabashi *et al.*, 2005).

The microfibrils
Three large glycoproteins known as microfibrils have been identified: fibrillin-1, fibrillin-2 and fibrillin-3, of which fibrillin-1 and fibrillin-2 have been studied (Wagenseil *et al.*, 2007). Studies have suggested that fibrillin-1, apart for being a prerequisite for elastic fibre assembly, directly signals cells through cell-surface receptors and interacts with growth factors such as TGF-β (Chaudhry *et al.*, 2007). It has been proposed that decorin is involved in fibrillin-1 synthesis, but also binds to the molecule in the ECM (Trask *et al.*, 2000; Schaefer *et al.*, 2007). Mutation in the gene encoding fibrillin-1 leads to Marfans' syndrome in humans, and mice in which this gene has been deleted die within two weeks of birth from cardiac and lung disorders, including aortic aneurysms (Wagenseil *et al.*, 2007). Fibrillin-2-deficient mice on the other hand are healthy with regard to heart, vessel and lung function, but suffer from syndactyly. Mice with both genes deleted die in utero. The mixing of fibrillin-1 and fibrillin-2 deletions indicates that fibrillin-1 can somewhat compensate for fibrillin-2 and that fibrillin-2 is active earlier in the foetal life assembly than fibrillin-1.

Elastin associated proteins and the elastic fiber assembly.
The microfibril associated proteins (MAGP-1, MAGP-2), the fibulins (1-5) and EMILIN-1 are the main proteins associated with elastic fibre assembly (Wagenseil *et al.*, 2007). The lysyl oxidase (LOX) family; LOX and four LOX-like proteins (LOXL1-4) are responsible for cross-linking in both elastin and collagen.
MAGP-1 binds to both tropoelastin and fibrillin-1, thereby prompting the suggestion that it is a bridge between the two. However, studies of deficient mice indicate that it is not considered essential for normal elastic fiber assembly.
Fibulin-1 associates with basal membranes and the elastin core, but not with microfibrils. When the fibulin-1 gene is deleted, defects appear in lung, kidney and capillaries but not heart or aorta suggesting importance in basement membrane organization, angiogenesis and capillary formation. Fibulin-2 binds to the basement membrane, tropoelastin and fibrillin-1. It does not associate to any human condition and gene-deleted mice remain healthy. Fibulin-3 interacts weakly with tropoelastin, has been detected in capillaries and is associated with macular dystrophy. Fibulin-4 binds to fibrillin-1 but moderately to tropoelastin. Mutation in the human gene for fibulin-4 is linked to a form of cutis laxa, a condition presenting loose skin, emphysema, aortic tortousity and ascending aneurysms. Gene-deleted mice die perinatally from severe vascular and lung defects and animal studies indicate that fibulin-4 facilitates cross-linking of elastin. Fibulin-5 binds cell surface integrins and tropoelastin and has a weak affinity to fibrillin-1. It is also seen in the elastin microfibril interface. Fibulin-5 gene mutation also induce cutis laxa and the gene-deleted mice phenotype resembles that of the fibulin-4 gene deleted mice, but less severe than the latter and live a normal lifespan.

EMILIN-1 (elastin microfibril interface located protein) has bindings to elastin and fibulin-5. EMILIN-1-deficient mice live normally but their elastic fibres are thinner and their TGF-β signalling is higher. Early elastic fibre assembly in mice starts with up-regulation of fibrillin-2 and MAGP-1 before day 14 of embryonal life, ending by the time of delivery. The subsequent up-regulation of all other proteins involved starts at embryonal day 14, continues until post-partum day 14, decreases until post-partum day 60 and remains steady until day 180.

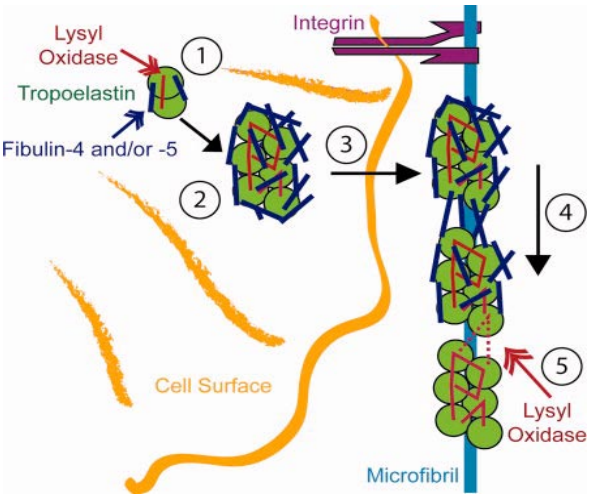


Fig 13. *Elastic fiber assembly. (1) Tropoelastin transported to assembly sites on the plasma membrane where it is organaized into small aggregates. The aggregates are cross-linked by LOX , facilitated by fibulin-4 and/ or fibulin-5 which are also possibly limiting the size of the aggregates. (2) The aggregates remain on the cell surface while newly secreted elastin is added. (3) The aggregates are transferred via cell surface integrins to extracellular microfibrils assisted by fibulin-4 and/ or fibulin-5. (4) Elastin aggregates on the microfibril coalesce into larger structures wich may be facilitated by fibulin-4/ fibulin-5. (5) Further cross-linking by LOX to complete elastic fiber.*

The degradation of the elastic fibers is slow in healthy individuals due to the extensive cross-linking. If damage of the elastic tissue occurs by injury, disease or aging an excessive or aberrant remodelling is initiated which leads to compromised mechanical properties of the affected tissues (Mithieux *et al.*, 2005).

Sex steroid hormone receptors

Sex steroid hormones are synthesized in ovaries, placenta, testes or the adrenal cortex (Beato *et al.*, 2000). They are, like the other two steroid hormone categories glucocorticoids and mineralocorticoids, derived from cholesterol. They are lipophilic, steroid molecules transported in blood bound to their specific binding protein SHBG. At the target cell they pass the cell membrane by diffusion. In the cell they are bound to their specific NRs, proteins which are key mediators of hormonal effects. As an effect of this specific and high affinity ligand binding, the NRs can stimulate or suppress gene expressions by: 1) relaying activating or repressing signals to the gene translational system or 2). protein-protein interactions or 3) integration in the intracellular signalling network. The receptors of thyroid hormone, retinoic acid and more than 60 known and unknown ligands are members of the NR family. The known sex steroid hormone receptor isoforms or subtypes are described below:

Estrogen Receptor
The two oestrogen receptor isoforms are cloned from different genes, ER-α and ER-β (Walter *et al.*, 1985; Kuiper *et al.*, 1996). The effect of ER-α is proliferative on endometrium, breast, squamous cells and fibroblasts (Kanda *et al.*, 2005; Mariotti, 2005). An additional anti-inflammatory effect on fibroblasts is found, but results concerning the effect on collagen synthesis are contradictory. ER-β is expressed in different tissues, but the only synergistic effect of ER-α and ER-β known is on bone preservation (Zallone, 2006). No other specific stimulating effect of ER-β is reported.

Progesteron receptor
PRs exist in two subtypes, PR-A and PR-B (Vegeto *et al.*, 1993). They derive from the same gene and are identical except that PR-B contains 164 additional amino acids. PR-B is a strong gene activator in many target cells, while PR-A is a dominant transcription suppressor of PR-B and others e.g. ER-α. As an effect of this complexity, progesterone can act as stimulator and suppressor of proliferation simultaneously in different tissues of the same organ (Fu *et al.*, 2003). Collagen degradation may be suppressed by progesterone, but the effects on ECM decrease after menopause as the number of PRs declines (Robinson *et al.*, 2003; Kanda *et al.*, 2005).

Androgen receptor
In concordance with PRs, ARs occur in two subtypes AR-A and AR-B. AR-B contains 187 additional amino acids. It is transcription-activating while AR-A is suppressive. However, AR-A is expressed at substantially lower levels than AR-B and its androgen action contribution is not known (Beato *et al.*, 2000; Liegibel *et al.*, 2003). Androgenic effects mediated by ARs are anabolic and hence stimulate collagen synthesis and inhibit degradation (Shin *et al.*, 2005). A pro-inflammatory effect has also been seen (Kanda *et al.*, 2005).

Aging of ECM

Since the ECM is constantly remodelled and the reactions and property demands are tissue-specific, adequate comparisons are important in this field of research. Age influences ECM metabolism and should be taken into account when comparing patient groups. Although most age studies are performed on skin, vascular tissue, bone or cartilage, age changes in pelvic floor ECM may be presumed.
In older age cell turnover slows (Freemont *et al.*, 2007). Apoptosis, which normally occurs in the ECM becomes dysregulated and increases with age.
Collagen turnover decreases and collagen molecules develop additional non-enzymatic cross-links. This results in an increase in collagen concentration and decreased solubility (Yamauchi *et al.*, 1988). These cross-links also affect the already extensively cross-linked elastic fibres, and the resulting collagen and elastin property changes result in increased tissue stiffness. Moreover, signs of impairment by age of the elastin-laminin receptor pathway have been discovered in vitro studies (Fulop *et al.*, 2001). In aging human skin, there is a decrease in large proteoglycans but an increase in a modified form of decorin (Carrino *et al.*, 2003). This form, named decorunt, is decorin without its C-terminal and thereby unable to bind to collagen as extensively as decorin does. This alters the collagen assembly and suggests a specific age-related catabolic pathway for decorin.

Pelvic floor extracellular matrix

Studies of the pelvic floor extracellular matrix started in the late 1980s and interest has increased as new experimental methods have been introduced. These studies are hard to compare since biopsy sites differ and most researchers include the epithelium, for which reason the important underlying

tissue representative of the endopelvic fascia is not adequately elucidated. A hormonal effect on the vaginal squamous cell tissue has been established, so that differences in hormonal status or treatment would potentially interfere more when the mucosa is not excised (Furuhjelm *et al.*, 1980). To be able to differentiate the ECM changes specific for POP and SUI, respectively, it is of utmost importance that patient groups are "clean" and that controls are free of pelvic-floor dysfunctions.

Pelvic Organ Prolapse

Pelvic organ prolapse (POP) has been studied on prolapsed full-thickness vaginal wall or supposedly stretched uterosacral or cardinal ligaments. In both these cases the results are hard to interpret as being cause of or effect of the damage to the POP.

In 1996 Jackson et al published a study of collagen, elastin and MMPs in vaginal cuff in women undergoing vaginal hysterectomy in POP patients and controls (Jackson *et al.*, 1996). The investigation showed an increase in collagen content and mature cross-links combined with an increase in MMP activity, but no difference in collagen I : III ratio or in desmosine as a reflection of elastin. A later study confirmed collagen increase, but found an additional increase in collagen III and a more limited increase of MMPs in POP (Moalli *et al.*, 2005). One study of paraurethral tissue however, showed an increase in markers of collagen break-down (Edwall *et al.*, 2008).

Not surprisingly, several studies of smooth muscle content in vaginal wall have shown a decrease in the stretched POP tissue (Boreham *et al.*, 2002; Boreham *et al.*, 2002; Badiou *et al.*, 2008).

An immunohistochemical study of cardinal ligaments revealed an increase in collagen III, decrease in elastin and increase in tenascin, a marker of ECM damage in POP (Ewies *et al.*, 2003). The results concerning elastin and tenascin were verified in a later study of the uterosacral ligaments (Goepel, 2008).

The most interesting findings are in animal studies where mice deficient in both elastin associated proteins fibulin-5 and LOXL1 developed POP during pregnancy or after giving birth (Liu *et al.*, 2006; Drewes *et al.*, 2007). To evaluate these results, the vaginal wall in normal pregnancies of wild-type mice was studied and a remodelling of the elastic fibres was discovered (Drewes *et al.*, 2007). During pregnancy the content of fibulin-5, to some extent tropoelastin and LOX mRNA expression, decreased. The day before parturition, LOX started to up-regulate, and this continued after birth. A burst in fibulin-5 expression and an increase in tropoelastin ensued 12- 24 hours post-partum, followed by declining levels for seven days. Desmosine content increased after the fibulin-5 burst up to seven days after delivery. LOXmRNA increased after parturition, but LOXL1 remained unaffected. In summary, that study showed that the vaginal ECM is remodelled in mice during pregnancy, delivery and postpartum period: this requires intact fibre assembly ability.

Stress urinary incontinence

When the TVT® procedure was introduced in the 1990s, tissue research started in the para-urethral connective tissue, since this was surgically available and considered representative of the endopelvic fascia (DeLancey, 1994). Different methods are used for quantification and, as in POP, the epithelium may sometimes interfere with the results.

Falconer et al found an increased collagen concentration, decreased solubility indicating more mature cross-links and wider collagen fibrils in paraurethral biopsies of pre-menopausal SUI women compared to controls (Falconer *et al.*, 1998). No clear differences were seen regarding the postmenopausals (Falconer *et al.*, 1998). Another indication of more mature cross-linked collagen in SUI was decreased collagen markers in tissue, serum or urine (Kushner *et al.*, 2004) (Edwall *et al.*, 2005) .On the other hand some studies show decreased collagen in paraurethral biopsies, especially concerning collagen III in SUI (Keane *et al.*, 1997; Bakas *et al.*, 2004; Lin *et al.*, 2005), but also unchanged collagen mRNA and protein expression (Bakas *et al.*, 2004). Polymorphism in the collagen α-1 chain has also been associated with SUI (Skorupski *et al.*, 2006).

Concerning the SLRPs studied in para-urethral biopsies, up-regulation of decorin mRNA and down-regulation of fibromodulin mRNA related to menstrual phase in pre-menopausals were discovered, but also no significant difference between SUI and controls in another study (Falconer *et al.*, 1994; Falconer *et al.*, 1998; Lin *et al.*, 2005; Wen *et al.*, 2007) At protein level a recent study reported a menstrual-phase-dependent increase in expression of decorin and biglycan and decreased expressions of fibromodulin, as opposed to no difference in earlier studies(Falconer *et al.*, 1998; Falconer *et al.*, 1998; Wen *et al.*, 2007) .

Paraurethral elastic fiber associated proteins have been investigated in relation to pre-menopausal SUI. Fibrillin-1 mRNA was up-regulated compared to controls, but no increase was found in the corresponding protein expression (Wen *et al.*, 2006). Fibrillin-2 and fibrillin-1-related TGFβ1 remained unchanged, while a TGFβ binding protein, LTBP1, showed menstrual-phase-dependent changes.

In summary, the above-related data all point towards the insight that differences in ECM composition can be prerequisites for the development of pelvic-floor dysfunction. The diversity in results warrants studies of “clean” patient groups, considering the normal age changes and standardized biopsy procedure.

Aims of the study

The basic aim of work reported in this thesis was, through study of the endopelvic extracellular matrix, to find out why some women develop pelvic floor dysfunction after vaginal delivery. The hypothesis was that constitutional impairments result in pelvic organ prolapse or stress urinary incontinence and that these conditions are considered to be two separate entities. In a longer perspective increased knowledge at tissue level can lead to clarification of treatment failures and the development of new alternatives.

Specific aims were:

- to analyze differences in collagen concentration and extractability by pepsin digestion in women suffering from *pelvic organ prolapse* compared to healthy controls.

- to analyze differences in gene expressions of the common collagen types, the most frequent small leucine-rich repeat proteoglycans important for collagen cross-linking and representatives of two molecular families involved in the elastic fiber assembly in *pelvic organ prolapse* compared to healthy controls .

- to analyze differences in gene expressions of the common collagen types, the most frequent small leucine-rich repeat proteoglycans important for collagen cross-linking and representatives of two molecular families involved in the elastic fiber assembly in *stress urinary incontinence* compared to healthy controls.

- to analyze the presence of sex steroid hormone receptors and their gene expressions in *stress urinary incontinence* and compare to healthy controls .

Materials and Methods

SUBJECTS

The biopsies from women suffering from POP (I, II) and SUI (III, IV) were collected at the time of surgery for the respective condition. In studies I and II POP was defined as prolapse stage II or more according to POP-Q grading system (Bump *et al.*, 1996). In studies III and IV SUI was defined following a standardized urogynaecological clinical evaluation performed to consider surgical treatment. As controls served women undergoing surgery for other benign conditions such as myomas, ovarian cysts and irregular bleeding (I-IV).

The patients and controls were matched for age and parity and divided into sub-groups. In paper I the patients (n=22) and controls (n=13) were divided at the age of 53, the median age of all participants in the paper.

In paper II the biopsies from women with POP (n=6+5) and from controls (n=8+6) were divided by menopausal status. In paper III, by analogy with study II, SUI (n=12+12) and control (n=8+6) pre- and post-menopausal sub-groups were formed.

In the immunohistochemical study (paper IV) the samples from women suffering from SUI (n=12) were divided into a pre-menopausal (n=4) and a postmenopausal (n=8) group, similar to controls (n=6+5). Corresponding divisions in SUI (n=7+7) and control sub-groups (n=5+5) were applied in the real-time RT-PCR study (paper IV).

SAMPLING PROCEDURE

From the participants in all studies punch biopsies were obtained from the paraurethral ligaments 5 mm from the urethral orifice, fig 14. The biopsies were 6 mm in diameter and 10 mm in depth and weighed 20-40 mg.

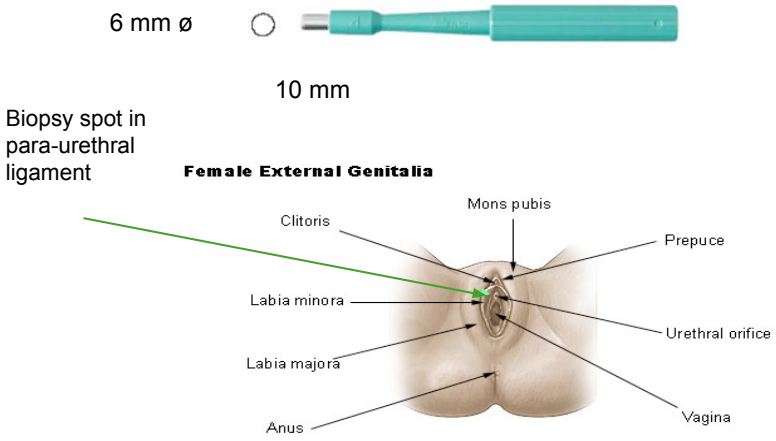


Fig 14. *Biopsy technique*

The mucosa was excised, except when collecting biopsies for immunohistochemistry (I-IV). The biopsies for the hydroxyproline analyses and initially for real-time RT-PCR were immediately frozen in liquid nitrogen (I, IV). The remaining biopsies analyzed with real-time RT-PCR were fixed in RNA-later® (Ambion, TX, USA) (II-IV) when available. The latter method is considered equally effective for fixation, but is easier to manage when collecting biopsies in a clinical situation. After fixation these biopsies were stored at -70ºC. The biopsies prepared for immunohistochemistry were fixed in 4% formaldehyde, followed by dehydration in 70% ethanol and paraffin-embedding.

BIOCHEMICHAL ANALYSIS (I)

Hydroxyproline is one of the most frequently found amino acids in collagen and its content is considered to correspond to the collagen concentration (Stegemann *et al.*, 1967).

Collagen concentration

The biopsies were homogenized and extracted by 0.5 M HAc. After hydrolysis in 6 M HCl the hydroxy-proline concentration was estimated using spectrophotometry.

Extractability by pepsin digestion

Pepsin was added and the collagen concentration was estimated as hydroxyproline in the extractable part as corresponding to non–cross-linked collagen (Stegemann *et al.*, 1967). Since pepsin contains some hydroxyproline, this amount was subtracted from the result.

IMMUNOHISTOCHEMISTRY (II-IV)

The biopsies were sectioned, mounted on glasses and stained. In paper IV the avidin-biotinylated (ABC)-peroxidase complex method was used, while the MACH3™ Mouse/Rabbit –Probe HRP Polymer Kit (Biocare Medical, CA, USA) was employed in papers II-III. The staining reaction was developed using DAB in paper IV (Vector, Burlingame, CA, USA) and II-III (Biocare Medical) and all specimens were counterstained in 10% Mayer´s haematoxylin solution.

Mouse monoclonal primary antibodies against ER-α, ER-β, PR (A+B), PRB and AR were used in study IV and against collagen I, collagen III, decorin, fibrillin-1 and fibulin-5 in papers II-III. The antibodies detecting fibromodulin and lumican were rabbit polyclonal (II-III). In sections used as negative controls all steps were performed omitting the primary antibody.

The manual scoring of positive intracellular staining 0-3+ was performed by two independent researchers (IV). In addition, for ER-α and ER-β, image analysis was employed (IV). By using computerized colour discrimination (Colorvision software, Leica Imaging System Ltd., Cambridge, UK) in systematic randomly selected fields, the total area of positively-stained cells was measured and expressed as a ratio of the total area of cells.

REAL-TIME RT-PCR (II-IV)

The biopsies were homogenized frozen using a dismembranation apparatus (Reutsch KG, Haan, Germany) achieving a fine powder from which the total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was controlled for by an OD_{260}/OD_{280} ratio >1.7 (Eppendorff Bio Photometer) in the samples included, supplemented by electrophoresis on 1.5 agarose gels to visualize ethidium-bromide-stained RNA in ultraviolet light. The RNA was stored at

-70ºC until the reverse transcription to cDNA was performed by incubating with Superscript™Rnase H-reverse transcriptase. After storing at -70ºC the cDNA was mixed with Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and placed in a 96-well optical PCR plate in triplicates with adequate purchased probes and primers (Taqman® gene expression assays, Applied Biosystems) encoding ER-α, ER-β, PR, and AR in paper IV and collagen I, collagen III, decorin, lumican, fibromodulin, fibrillin-1 and fibulin-5 in papers II-III. In addition, ribosomal 18S was analyzed in triplicates as internal standard (II-IV). Using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems) the real-time PCR reaction was carried out involving 40 cycles of denaturation-annealing according to a standard manufacturer's protocol. The threshold cycles, at which an increase in reporter fluorescence above the baseline signal was first detected, were determined for the investigated genes in every sample. mRNA expressed by a C_t value >40 was considered non-detectable. The C_t values for the respective genes were subtracted from the 18S C_t in each sample, giving the ΔC_t. As a high C_t value corresponds to a low mRNA level, the mRNA expressions were presented inverted as $10/\Delta C_t$. As controls for primers in paper IV, purchased total RNA from testicular and ovarian tissue was used.

STATISTICAL ANALYSES

In paper I descriptive statistics implied interaction of age concerning collagen concentration, i.e. age affected collagen concentration differently in different age groups. This was verified using multiple regression and two-way ANOVA. Since Levene's test showed homogeneity of variance, comparisons between patients and controls were performed with the univarate test of significance for planned comparison in the below-53 and above-53 age groups. There was no interaction of age concerning extractability by pepsin digestion and patients and controls were compared using a 2-way ANOVA.

In papers II and III, descriptive statistics of the real-time RT-PCR results implied interaction of age concerning some variables. To investigate this in the respective pre- and postmenopausal groups, each variable was analyzed using univariate tests of significance comparing inclinations of parallel lines and ANCOVA test with age as co-variant. In variables where there was a parallel down-regulation by age among both patients and controls, a comparison was performed between the total patient- and control-groups irrespective of menopausal status, using ANOVA. When an interaction of age was discovered, patients and controls were divided into pre- and post-menopausal groups and compared to their respective control group with the simple main effects test.

In paper IV the Mann-Whitney test was used for comparing patients and controls in their pre- and post-menopausal groups.

Result

PELVIC ORGAN PROLAPSE

Collagen (I, II)

In paper I a 30 % lower collagen concentration was discovered in women below 53 years than in matched controls, p=0.01, fig 15. No corresponding difference among the older women was seen, nor was there a difference in extractability by pepsin digestion between patients and controls in any age group. The part extractable by pepsin digestion decreased significantly by age irrespective of POP (p=0.001).

Paper II revealed no significant difference in the gene expression of collagen I or III between women suffering from POP and controls. Immunohistochemically, collagen I and III were both well expressed, covering large areas of the ECM. No obvious difference was seen between the groups, but random variations of staining intensity and distribution within these small biopsies did not allow systematic scoring.

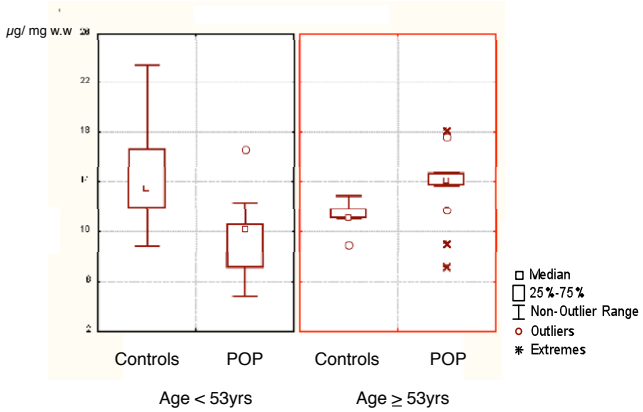


Fig 15. *Collagen concentration in controls and women with POP.*

Small Leucine-rich Repeat Proteoglycans (II)

The most dramatic changes in small leucine-rich repeat proteoglycans were found in the pre-menopausal POP group, where there was a 16-fold decrease in mRNA expression of the gene encoding decorin, p= 0.0001, and an 8-fold decrease of lumican expression, p=0.001, compared to pre-menopausal control, fig 16, fig 17. An additional significantly lower expression of fibromodulin mRNA appeared among all women suffering from POP compared to all controls, p=0.004, fig 18. When decorin expression was studied with immunohistochemistry the sections from the pre-menopausal women with POP clearly demonstrated weaker immunoreactivity than pre-menopausal controls did. Lumican and fibromodulin were both adequately expressed, but random variation within these small biopsies did not allow scoring.

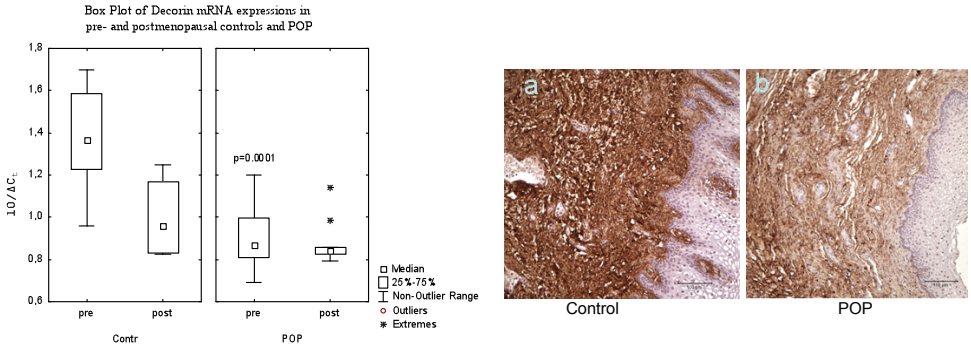


Fig 16. *Box plot of decorin mRNA expression and immunohistochemical staining of decorin (brown colour) in pre-menopausal controls and POP.*

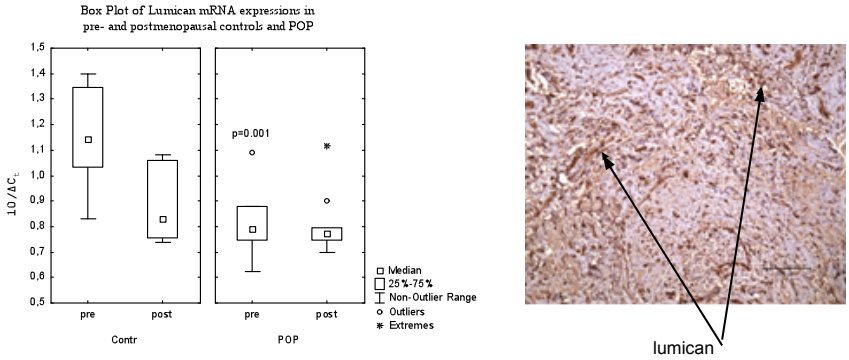


Fig 17. *Boxplot of lumican mRNA expression in controls and POP and immunohistochemical staining of lumican (brown colour)*

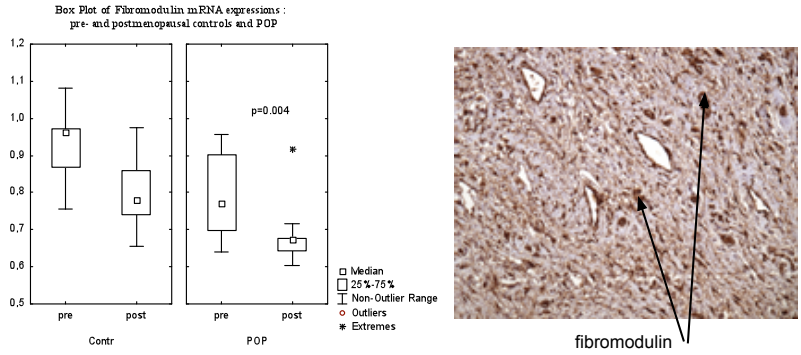


Fig 18. *Boxplot of fibromodulin mRNA expression in controls and POP and immunohistochemical staining of fibromodulin (brown colour)*

Elastin Associated Proteins (II)

Of the investigated elastin associated proteins fibulin-5 mRNA was lower expressed in women with POP compared to all controls, p= 0.001, while there was no difference in fibrillin-1 mRNA expression, fig 19.
Immunoreactivity for fibulin-5 revealed randomly distributed small areas of proteins with variations within the groups, why no group difference could be seen.

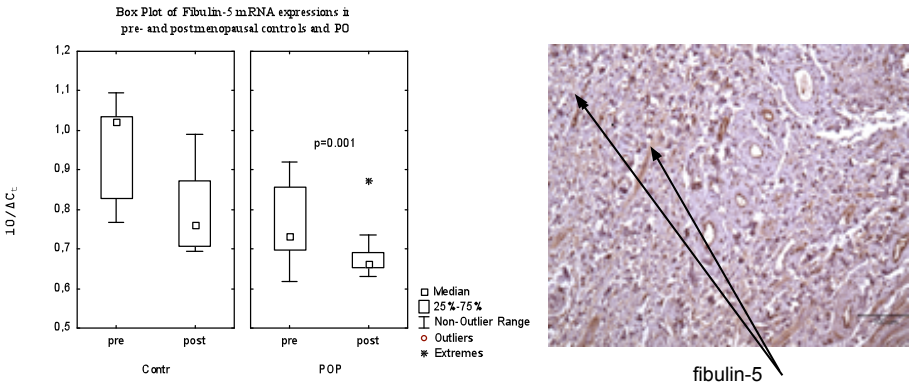


Fig 19. *Boxplot of fibulin-5 mRNA expression in controls and POP and immunohistochemical staining of fibulin-5 (brown colour)*

STRESS URINARY INCONTINENCE

Collagen (III)

There was no significant difference in mRNA expression of collagen I or III between women suffering from SUI and controls before or after menopause.
Immunohistochemically collagen I and III were well expressed in all groups..

Small Leucine-rich Repeat Proteoglycans (III)

None of the studied proteoglycans decorin, lumican or fibromodulin did differ in mRNA expression in relation to occurrence of pre- or post-menopausal SUI.
When performing immunohistochemistry the three SLRPs were widely distributed in the ECM.

Elastin associated proteins (III)

A significant decrease in fibrillin-1 mRNA expression compared to controls was discovered in women suffering from SUI irrespective of menopausal status, p=0.03, fig 20. This was verified at protein level by immunohistochemistry as fibrillin-1 was more frequent and displayed more intense staining, particularly when biopsies from pre-menopausal women with SUI were compared to those from pre-menopausal controls.

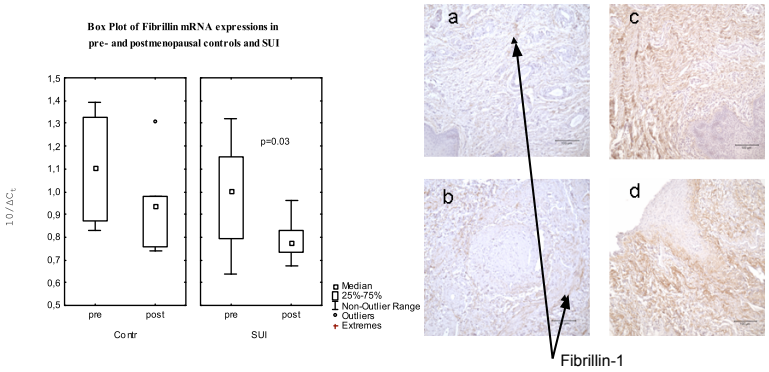


Fig 20. *Box plot of fibrillin-1 mRNA expression in and controls and SUI and immunohistochemical staining of fibrillin-1 (brown colour) in a. pre-menopausal control b. postmenopausal control c. pre-menopausal SUI d. postmenopausal SUI*

There was no difference between SUI and controls in gene signal for fibulin-5 or protein expression by immunohistochemistry.

Sex steroid hormone receptors (IV)

ER-α, ER-β, PR-(A+B), PR-B and AR were all expressed with immunohistochemistry in the paraurethral ECM. Image analysis showed a significant increase in ER-β expression in pre-menopausal women with SUI compared to pre-menopausal controls, p=0.02, but no difference among post-menopausals or concerning ER-α in any group, fig 21. This was in line with the manual scoring, which also showed a decrease in PRs in patients and controls after menopause, fig 22.

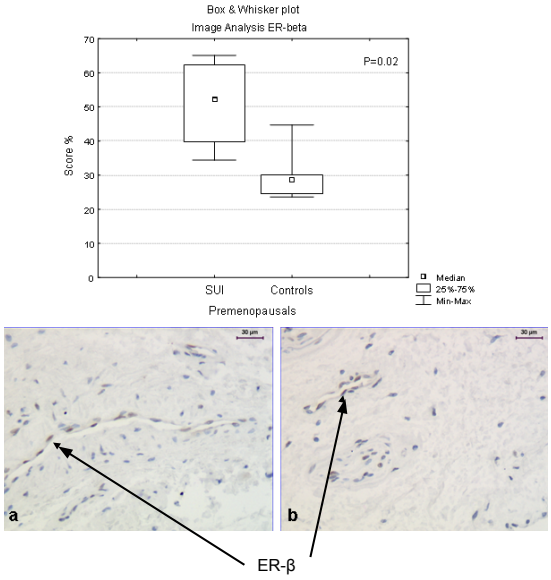
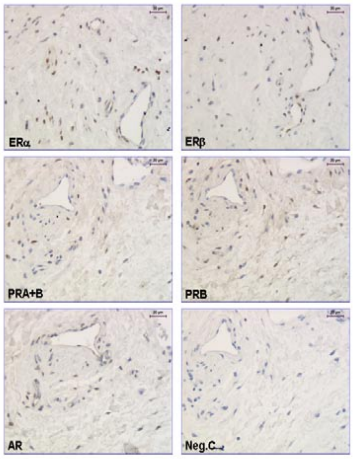


Fig 21. *Boxplot of ER-β expression by immunohistochemical image analysis and immunoreactivity (positively stained cells are brown and negatively stained cells blue) of a. pre-menopausal SUI and b. pre-menopausal control*



Group	ER-α	ER-β	PR (A+B)	PRB	AR
SUI pre-menopausal	2+ (2-3)	2+ (1-2)	3+ (2-3)	2+ (1-2)	2+ (1-3)
Control pre-menopausal	2+ (2-3)	1+ (1-2)	2+ (2-3)	2+ (1-2)	2+ (2-3)
SUI postmenopausal	2+ (2-3)	1+ (1-2)	2+ (2-3)	1+ (1-2)	2+ (0-2)
Control postmenopausal	2+ (2-3)	1+ (1-2)	2+ (1-3)	1+ (1-2)	2+ (2-3)

Fig 22. *Immunohistochemic identification of all investigated sex steroid hormone receptors (positively stained cells are brown and negatively stained cells blue) and table of manual scoring.*

Real-time RT-PCR exposed no difference in gene signalling regarding any NR between SUI and controls before or after menopause. ER-β was fully detectable only in five samples of 24, so that calculation for group differences regarding mRNA expression was not feasible. The PRs were significantly down-regulated by age in patients and controls, p=0.001, corresponding to the finding at protein level.

Age (II, III)

For all the investigated proteins, there was a considerable decrease in gene expression by age in controls, SUI and POP, except for decorin and lumican in the POP group where the mRNA expressions were low irrespective of age.

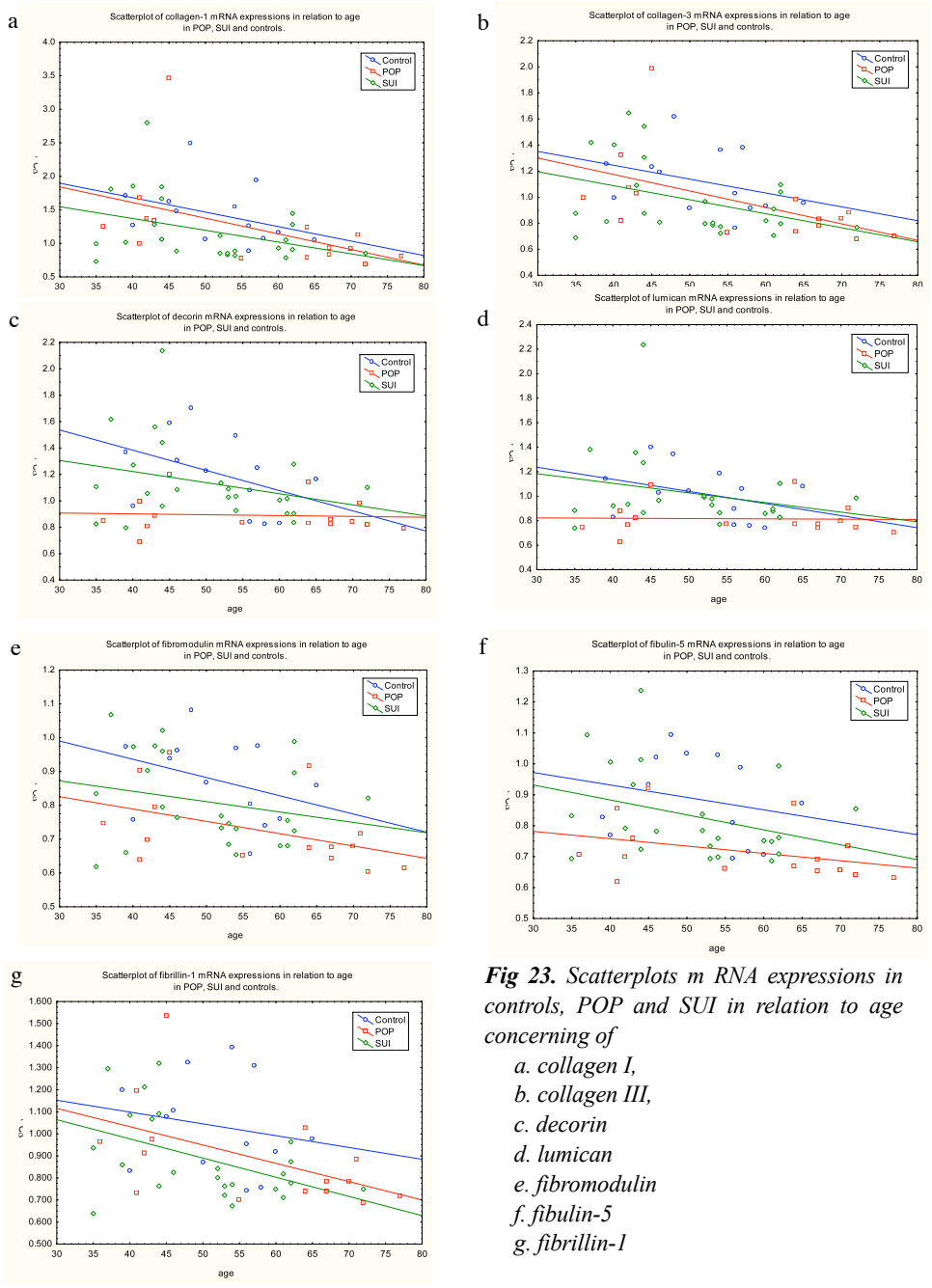


Fig 23. *Scatterplots m RNA expressions in controls, POP and SUI in relation to age concerning of*

a. collagen I,
b. collagen III,
c. decorin
d. lumican
e. fibromodulin
f. fibulin-5
g. fibrillin-1

Discussion

Pelvic floor dysfunction is a sizable health care issue for the community. In Sweden the costs for incontinence are approximatly 2-4 % of the total health care costs. The costs for POP surgery in the US 1997 were estimated to 1 billion $ which was equal to the costs for breast cancer surgery. POP surgery was furthermore found to be as frequent as cholecystectomy in women (Subak *et al.*, 2001). On top of this health care consumption, there are reasons to believe that many otherwise active women experience reduced quality of life without getting adequate attention from the medical services, partly due to the shamefulness of the symptoms, but also because of limited knowledge in the profession. This is confirmed by epidemiologic studies on symptoms from the pelvic floor in the population. They show that symptoms related to untreated pelvic floor dysfunctional conditions are not unusual already before menopause and common during the years around retirement from working life, when women in the western world are still leading a very active life (Tegerstedt *et al.*, 2005).

Pelvic floor dysfunction has received little attention in terms of research. It has been considered a part of the senescence and has been looked upon with a passive attitude. It is therefore of utmost importance that more knowledge about these conditions is gained so as to assure adequate treatment approaches.

As a result of basic science shortage in this area, little is known about the ECM of the human endopelvic fascia crucial for maintaining pelvic floor integrity under normal conditions. In mice, a remodeling of the vaginal ECM at the time of pregnancy, delivery and postpartum period has been discovered and it is not unlikely to posit a similar process in humans, considering the pronounced remodeling of the ECM in human cervix and uterus during this period (Granstrom *et al.*, 1989; Sennstrom *et al.*, 2000; Hjelm *et al.*, 2002; Drewes *et al.*, 2007). Apart from hypothetical initial constitutional variations in the pelvic floor ECM, there may be individual differences in ability to adjust to such a remodeling that could result in reduced tissue sustainability and pelvic floor dysfunction.

Given the predominant explanatory models for POP and SUI, it seems reasonable to suppose that the two conditions originate from different aberrations in the ECM (DeLancey, 1993; Ulmsten, 1997). In POP the tissue breaks and a herniation from an adjacent organ develops and in SUI there is an overextension of the anterior vaginal wall.

In the ECM two molecular complexes are giving the fibrous connective tissue its mechanical properties: collagens are responsible for tensile strength and the elastic fibers are contributing with elasticity and ability to recoil (Gelse *et al.*, 2003; Mithieux *et al.*, 2005). The SLRPs are essential for collagen cross-linking and thereby influencing collagen function; but the very abundant decorin also seems to be connected to the elastic fiber assembly (Svensson *et al.*, 2000; Trask *et al.*, 2000; Kalamajski *et al.*, 2007; Kalamajski *et al.*, 2007; Schaefer *et al.*, 2007; Kalamajski *et al.*, 2008; Kalamajski *et al.*, 2008).

In the work presented in this thesis we confirmed our hypothesis of POP and SUI being different conditions in terms of dysfunction on the tissue level. The alterations found in POP seems mainly related to collagen and collagen cross-linking and SUI to elastic fiber assembly.

Moreover are the results substantiating that POP in younger years before menopause is more severe not only from a clinical poiny of view, but also reflected in the ECM. In the biopsies from younger women suffering from POP the mRNA expressions of all the investigated SLRPs - lumican and fibromodulin, and in particular decorin-were lower, which is likely to affect collagen cross-linking and fibril assembly (paper II). From these results an impairment of collagen function can be anticipated. This could also affect the collagen turn-over and result in the lower collagen concentration found in paper I, although

not reflected by changes in collagen gene expressions (paper II). Decreases in collagen and collagen breakdown have been confirmed by other researchers, but the coincidences with SLRP regulation is registered for the first time in the works of this thesis (Jackson *et al.*, 1996; Moalli *et al.*, 2005; Edwall *et al.*, 2008).

One author has described the decorin expression by real-time RT-PCR in 3 pre-menopausal women compared to 7 pre-menopausal controls in vaginal wall and in contrast found decorin mRNA significantly up-regulated in POP (Song *et al.*, 2007). This study can however be criticized for several reasons apart from the small size of the study. The epithelium was left on the biopsies interfering with stromal cell activity and the biopsies were supposedly obtained from a prolapsed part of the vagina, why the increased mRNA expression can be a part of response to tissue damage by the stretch itself, rather than involved in the mechanism causing POP (Hakkinen *et al.*, 1996; Provenzano *et al.*, 2005). Furthermore can including as many as 45 replicate cycles and the use of β-actin be questioned. When choosing the adequate housekeeping gene it should be more expressed than the investigated genes. This was not the case in our study on the cell poor stromal tissue, why ribosomal 18S was considered a better choice than β-actin .

In all women suffering from POP irrespective of age or menopausal status more moderate, but significant decreases of fibromodulin and the elastic fiber associated fibulin-5 mRNA were discovered. These findings, related to both collagen and elastin assemblies, can be interpreted as a basic pre-requisite for all POP patient, but with additional SLRP down-regulations among the younger.

In animal studies, mice with deleted genes for fibulin-5, but also for the cross-linking enzyme LOXL1, developed prolapse at parturition, which supports our findings concerning fibulin-5 and the cross-linking defect hypothesis (Liu *et al.*, 2006; Drewes *et al.*, 2007). In an investigation of human utero-sacral ligaments there was decreased mRNA expression of LOX, LOXL1 and LOXL2, but an increased expression of fibulin-5 mRNA (Klutke *et al.*, 2008). It is however hard to interpret these results, since there is no information provided of ages of the included women and the ligaments were assumed to have been damaged by the stretch.

Expression of the investigated proteins were clearly confirmed in the immunohistochemical part of paper II, but could not be scored since the biopsies were irregular and showed wide variations of expressions even within the same biopsy. Concerning decorin however, there was an striking reduction in protein expression between pre-menopausal POP and pre-menopausal controls in accordance with the lowered gene signal.

For the first time we show that a decrease in fibrillin-1 mRNA expression, a microfibril vital for the elastic fiber assembly, was associated with SUI (paper III). Fibrillin-1 is central in the assembly process, why even the limited, but significant, decrease in SUI compared to controls seen in our study potentially could diminish the elastic properties resulting in hypermobility of the pelvic floor (Mithieux *et al.*, 2005; Wagenseil *et al.*, 2007). Interestingly, was fibrillin-1 the only protein, except the collagens, in which the mRNA expression did not differ between patients with POP and controls. Additionally, none of the alterations discovered in POP reappeared in SUI, all findings substantiating the theory of POP and SUI as separate conditions.

An earlier study by Falconer at al reported increased collagen concentration and decreased extractability by pepsin digestion in pre-menopausal SUI with a corresponding collagen I and III mRNA increase and wider collagen fibrils seen in electron- microscope (Falconer *et al.*, 1998). A connection between our results could however be sought in the cross-linking process since fibrillin-1 and decorin do interact (Trask *et al.*, 2000; Schaefer *et al.*, 2007).

We could confirm the difference in fibrillin-1 regulation in SUI at protein level, but further studies are needed, involving more interactions in the complex elastic fiber assembly and cross-linking, especially since an opposing fibrillin-1 mRNA increase but unchanged protein expression in pre-menopausal SUI was reported recently (Wen *et al.*, 2006).

By studying the mRNA expressions of the ECM proteins in paper II and III a consistent decrease in expression by age was discovered among the controls, fig 23. A similar slower cell activity and turnover in the ECM as in other tissues is the probable explanation, now seen for the first time in pelvic floor endopelvic fascia (Freemont *et al.*, 2007). The inclination of lines of the POP related proteins are converging by age, which may confirm that age related tissue changes be additionally involved in POP development among the eldest. Interestingly, this was not the case concerning fibrillin-1 where the lines were diverging. These observations agree with epidemiology showing SUI due to hypermobility often starts earlier than POP and is not related to age per se.

In paper IV all the investigated sex steroid hormone receptor isoforms and subtypes were for the first time identified coinciding in the para-urethral stromal ECM. In earlier investigations of vaginal stroma the ER isoforms and PRs were recognized, but not the PRs differentiated into subtypes or the AR (Hodgins *et al.*, 1998; Fu *et al.*, 2003).

An increase of ER-β protein expression was seen in pre-menopausal SUI, but no other differences at mRNA or protein levels. In another imunnohistochemical study of ER isoforms of vaginal wall including epithelium, the expressions did not differ between SUI and control concerning any isoform, but decreased after menopause (Fu *et al.*, 2003). These results are hard to compare to ours since the epithelial cells are outnumbering the stromal cells by far and generally extensively express the estrogen receptors.

We found a postmenopausal reduction of both PR subtypes by immunohistochemistry confirmed by a significant corresponding decrease in mRNA expression of PRs, $p= 0.001$, irrespective of SUI. This finding is in line with earlier research and a reduction of the ER suppressing PRs could potentially increase the effect of the declining estrogen levels after menopause in this tissue (Conneely *et al.*, 2000; Robinson *et al.*, 2003).

The ARs have been identified before in genital skin, striated and smooth pelvic floor muscle cells and fibroblasts of cardinal ligaments, but its occurrence in para-urethral fibroblasts found in our study is new (Hodgins *et al.*, 1998; Ewies *et al.*, 2004; Ho *et al.*, 2004). Interestingly, the ARs and ERs were co-expressed here, as opposed to striated pelvic floor muscles where ARs were well expressed but ERs were absent (Ho *et al.*, 2004).

Due to the receptor occurrence, it seems likely that pelvic floor ECM is somehow regulated by sex steroid hormones, but their actions and interactions, not least during pregnancy and parturition in this tissue, are not yet clarified. Furthermore is the consequence of the registered increase in ER-β expression in pre-menopausal SUI hard to relate, since its mediated effects are sparsely elucidated in most tissues.

In summary, the work of this thesis show for the first time evidence for POP and SUI being separate conditions based on different changes from the normal pelvic floor ECM composition. Another novel result was that POP in pre-menopausal women was found to be related to several highly significant alterations in the ECM, while the changes were more moderate in postmenopausal POP. Concerning SUI, the connection to fibrillin-1 regulation is potentially very interesting, since this protein is crucial for the elastic fiber assembly. As targets for possible hormonal actions all sex steroid hormone receptors are by this work found to be represented in the pelvic floor ECM.

Conclusion

The present hypothesis of pelvic organ prolapse and stress urinary incontinence deriving from different alterations in the pelvic floor ECM was confirmed. The results therefore suggest different pathophysiological backgrounds to these conditions at tissue level. Further, the greatest changes were found in the pre-menopausal women with pelvic organ prolapse, reflecting the severity of pelvic floor dysfunction in this group.

- The collagen concentration was 30 % lower in the pelvic-floor ECM of women younger than 53 years suffering from POP, than in age-matched controls.

- There were decreases in mRNA expression of all the investigated small leucine-rich proteoglycans and the elastin-associated fibulin-5 in pre-menopausal women with POP compared to pre-menopausal controls. The 16-fold reduction in decorin mRNA was the most prominent finding and a corresponding weaker protein expression was found with immunohistochemistry.

- Among the post-menopausal women suffering from POP there were significantly lower mRNA expressions of fibromodulin and fibulin-5 than in postmenopausal controls.

- There was a reduction in the mRNA expression of fibrillin-1, the most crucial microfibril in elastic-fibre assembly, in women with SUI, irrespective of menopausal status, compared to healthy controls.

- All the hormone receptor isoforms or subtypes investigated were expressed in the pelvic-floor ECM. ER-β was more expressed by immunohistochemistry in pre-menopausal women with SUI than in pre-menopausal controls, but no corresponding elevation in gene expression was discovered.

Future perspectives

The ideal goal would be to find risk markers for the two conditions studied, and thus be able to plan delivery mode and, already before the first delivery, obviate the risk of a subsequent severe pelvic-floor dysfunction. This would be an alternative to the up-coming trend of women demanding caesarean section for fear of pelvic floor dysfunction.

Following the present work more basic research is needed on regulations of collagen cross-linking and its applications to pelvic-floor ECM. Of the SLRPs, the role of biglycan, sharing the decorin-binding site on the collagen fibrils, needs to be investigated. Since a major reduction in decorin mRNA expression was seen in POP, more knowledge of its involvement in other ECM processes such as elastic-fibre assembly would be interesting.

As well as studying their respective key roles in the assembly of the elastic fibre, signs of interactions of fibrillin-1 and fibulin-5 in the ECM need to be further elucidated. TGF-β-mediated actions of fibrillin-1 under different conditions in pelvic floor ECM could be investigated, as could the role of fibulin-4 compared to fibulin-5.

An investigation of the events taking place in human pregnancy, delivery and pos-partum regarding the collagen and elastin molecular systems would be of special interest. A study of the effects of birth trauma and its restoration in the ECM, not only directly but also over a long term, would give added information. If a remodelling in the pelvic-floor ECM takes place in humans, a prospective study to find representative markers from the time of the first pregnancy onwards would be ideal, although hard to accomplish.

The tissue related circumstances of the problematic recurrent large POP in the anterior vaginal wall needs to be studied more, both to find prerequisites for its existence and to help prevent possible ECM degradation leading to recurrence. Research concerning decorunt, a form of decorin seen in elderly would be appropriate here.

Sammanfattning på svenska

Sämre bäckenbottenbindväv kan orsaka framfall och ansträngningsinkontinens

Framfall och urininkontinens vid ansträngning är de två vanligaste åkommorna som omfattas av begreppet bäckenbottensvaghet. Behandlingen är ofta kirurgisk och sammantaget med uroterapeutisk behandling och hjälpmedel medför dessa åkommor avsevärda kostnader för samhället. Det finns dessutom goda skäl att anta att skam och skuld fortfarande hindrar kvinnor att söka hjälp, varför ett stort mörkertal är sannolikt. På basis av enkät studier i befolkningen kan närmare en miljon kvinnor vara drabbade i Sverige.
Min avhandling fokuserar på molekylära förändringar i vävnaden som orsaker till framfall och ansträngningsinkontinens. Framfall innebär att någon av slidans väggar eller livmodertappen glider ut mot eller t.o.m. ut genom slidmynningen. Vid ansträngningsinkontinens läcker urin, utan föregående trängning vid ökat buktryck, det vill säga vid hosta, nysningar, hopp, lyft etc.
Epidemiologiska studier har visat att förlossning är den största kliniska riskfaktorn för båda tillstånden, men det förklarar inte helt varför en del kvinnor drabbas och andra inte. Det kan finnas anledning att anta att vävnaden klarar den påfrestning som en förlossning innebär olika bra. En tänkbar orsak är att dess sammansättning är olika, vilket kan vara ärftligt betingat.
Bäckenbotten är en muskelplatta, men insprängt i musklerna, runt om i hinnor och samlat i stödjande ligament finns bindväv. Bindväven är gles på celler, fibroblaster, som producerar de molekyler som finns i bindväven. Där ger de fibrer som är uppbyggda av kollagen vävnaden dess hållbarhet. Elastiska fibrer bestående av elastin och ett antal associerade proteiner ger vävnaden elasticitet. Som organisatörer fungerar små proteoglykaner som påverkar kollagenets tvärbindningar. Hormoner som östrogen och progesteron, men även testosteron kan också antas påverka bindvävens omsättning och kvalitet genom sina receptorer.
Har bindväven en annan sammansättning hos kvinnor som drabbas av framfall respektive ansträngningsinkontinens jämfört med kvinnor utan sådana besvär?
För att söka svaret på den frågan, har bindväv i form av vävnadsbitar tagits från bäckenbotten på kvinnor med anstängningsinkontinens eller framfall, och jämförts med bindväven från besvärsfria kvinnor.

I den första studien fann vi att kvinnor med framfall som diagnosticerades före menopaus hade 30% lägre kollagenkoncentration jämfört med friska kvinnor.

I den andra studien studerades det genetiska uttrycket för följande bindvävsmolekyler; kollagen I och kollagen III, de tre små proteoglykanerna decorin, fibromodulin och lumican samt 2 proteiner associerade med den elastiska fibern; fibrillin-1 och fibulin-5. Samtliga molekylers lokalisation studerades med färgade antikroppar, s.k. immunhistokemi.
Stora skillnader registrerades hos de yngre kvinnorna med framfall. De genetiska uttrycken för de tre proteoglykanerna och fibulin-5 var signifikant sänkta, mest för proteoglykanen decorin; 16-faldigt lägre jämfört med friska kvinnor. Hos de äldre kvinnorna med framfall var genuttrycket för fibulin-5 och fibromodulin lägre, men inte för decorin eller lumican. Alla molekyler kunde identifieras med immunhistokemi. Immunoreaktiviteten för decorin var dessutom klart lägre hos kvinnor med framfall jämfört med friska.

I den tredje studien studerades kvinnor med ansträngningsinkontinens på samma sätt som i ovanstående studie.
Vi fann en signifikant sänkning i genuttrycket för fibrillin-1 hos kvinnor med ansträngningsinkontinens oavsett ålder, där också immunhistokemi visade låg immunoreaktivitet för fibrillin-1 hos kvinnor med inkontinens jämfört med friska.

I fjärde studien identifierades östrogenreceptorerna (ER) -α och –β, progesteronreceptorerna (PR) A och B samt androgenreceptorn (AR) med immunhistokemi och deras genuttryck hos kvinnor med ansträngningsinkontinens och hos friska kvinnor.
Alla hormonreceptorerna identifierades i bäckenbottenbindväven. Signifikant fler celler visade immunoreaktivitet för ER-β hos inkontinenta kvinnor som inte passerat menopaus, jämfört med motsvarande friska kvinnor.

Sammanfattningvis har avhandlingen visat att:

- Framfall och ansträngningsinkontinens är relaterade till förändringar i bäckenbottenbindväven.
- Förändringarna är helt olika hos kvinnor med framfall repektive ansträngningsinkontinens.
- Mest uttalade förändringar identifieras hos yngre kvinnor med framfall med påverkan på fem olika bindvävsmolekyler.
- Ansträngningsinkontinens var relaterad till förändringar i den elastiska strukturen med sänkt produktion av ett viktigt elastinassocierat protein.
- Bäckenbottenbindväven kan påverkas av hormoner.

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