University of Southampton

Faculty of Medicine

Influence of racial differences or topical oestrogen on vaginal skin extracellular matrix components in women with pelvic floor dysfunction

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Abstract

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Stress incontinence (SI) is more common in white women (61%) than in black African women (27%). Previous studies have demonstrated that collagen XVII is increased and the collagen I:III ratio is decreased in continent black women compared to white suggesting better adhesiveness and elasticity in the tissue of black women. We decided to examine this concept further and analyse the elastin content in paraurethral vaginal tissue of black and white women with or without stress incontinence. A further study was set up to examine if topical oestrogen could increase elastin and change associated components of white women with pelvic organ prolapse (POP). Oestrogen treatment has been shown to increase pro-mmp2 and new collagen formation in SI women, therefore it seemed possible that such treatment could affect the collagen and elastin components of POP favourably. The clinical symptoms of POP and surgical outcome may also be improved.

The first study examined the elastin content by histochemistry in paraurethral vaginal tissue while the second study assessed how locally applied oestrogen (Vagifem) given over three months influenced the mRNA expression of MMP2, elastin and collagen XVII and also elastin, collagen I and collagen III protein content of vaginal skin from women with POP.

In the racial comparison study black controls showed a highly significant increase in elastin content compared with white controls ($p<0.01$). For SI to occur in black women a severe insult that reduces elastin production appears to be necessary as black women with SI showed significantly lower elastin content compared with black controls ($p<0.05$).

After application of topical oestrogen to white women with POP it was shown that mRNA for MMP2 is up-regulated ($p<0.01$) while that for ER alpha receptor is not ($p=NS$). The message for collagen XVII was down-regulated ($p<0.01$) while collagen I and III protein were increased significantly ($p<0.001$ in both cases). The mRNA for elastin was significantly increased ($p<0.05$) after treatment but the increase in elastin protein staining did not quite reach significance.

In conclusion, black women have higher elastin content in vaginal tissue compared to white and this may contribute to the lower incidence of SI in black women. Topical oestrogen over a short period has remodelling effects on key factors of the extracellular matrix of vaginal tissue. Although significant rises in elastin mRNA were shown but not protein, this treatment over longer term application could enhance some of the changes seen. This research provides evidence that black women have beneficial characteristics of vaginal skin that could resist SI and POP. Oestrogen treatment with refinements for white women could mimic black characteristics and alleviate POP symptoms.

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List of Abbreviations

17β-HSD3	17-beta-hydroxysteroid dehydrogenase
5-HT	Serotonin
ADAM-10	Metallopeptidase domain 10
ADAM-9	Metallopeptidase domain 9
AMV	Avian myeloblastosis virus
AP1	Activator Protein-1
APES	Aminopropyltriethoxilane
AR	Androgen receptor
BC	Black control
BS	Black stress incontinent
CCA	Contractural arachnodactyly
cDNA	Complementary DNA
COL17A1	Collagen 17 alpha 1
Ct	Cycle threshold
DBD	DNA binding domaine
dNTP	Deoxynucleoside triphosphates
ECM	Extracellular matrix
ERK1	Extracellular signal-regulated Kinase
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EVG	Elastin van Gieson
G3PDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GAG	glycosaminoglycan
GAPDH	Glycerinaldehyde-3-phosphat-dehydrogenase
HESR1	Human estrogen receptor 1
HHL	Histidino-hydroxylysino-norleucine
HIV	Human immundefficiency virus
HLKNL	Hydroxylysino-keto-norleucine
HMMP2	Human matrix metalloprteinase2
HRT	Hormone replacement therapy
IGF	Insulin like growth factor
IL-1a	Interleucin-1a

kDA	Kilo Dalton
LAMC1	Laminin gamma1 gene
LBD	Ligand binding domain
MeSH	Medical Subject Headings
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MRI	Magnet resonance imaging
mRNA	Messenger ribonucleic acid
NA	Noradrenalin
NR	Nuclear receptors
PCR	Polymerase chain reaction
PFD	Pelvic floor dysorder
PFE	Pelvic floor exercises
POP	Pelvic Organ Prolapse
P-QOL	Prolapse Quality of Life
PR	Progesterone receptor
Pro-TNF-a	Pro-Tumor necrosis factor-a
RT	Reverse Transcriptase
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SI	Stress incontinence
SLRP	Leucine rich repeat proteoglycans
SVAS	Supravalvular stenosis
TIMP	Tissue inhibitors of metalloproteinase
TOT	Transobturator tape
TVT	Tension free vaginal tape
WC	White control
WS	White stress incontinent
Δ-HLNL	Dehydroxylysino-norleucine

Chapter 1 :

Introduction

1.1 Background

This thesis examines the presence of elastin in periurethral vaginal tissue of women from different races and assesses any diverse changes occurring when associated with stress incontinence. It also investigates the influence of vaginal oestrogen on elastin and associated extracellular matrix components in vaginal tissue from women with pelvic organ prolapse.

The pathophysiology of stress incontinence and pelvic organ prolapse and comparison to the normal anatomy are also presented in this Introduction.

1.2 Normal Anatomy

The support apparatus in the female pelvis is derived from different tissues, anatomical layout and their cooperation. The pelvic diaphragm is the key structure to prevent pelvic organ prolapse. In combination with the uterine, ovarian and vesical ligaments it acts as a support unit for the pelvis and its contents. The pelvic diaphragm consists of interacting and connected muscles fanning out and so building a muscle plate.

The main muscle is the levator ani muscle. This muscle has been divided into three different parts:

1) pubococcygeal muscle,

2) ileococcygeus muscle,

3) puborectalis muscle

Further important muscles are

4) ischiococcygeus muscle

5) Obturator internus muscle

The pubococcygeus and ileococcygeus form the main part of the levator though the iliococcygeus, the most posterior part of the levator ani, is often poorly developed. They run backward from the pubic bone toward the coccyx and may be damaged during delivery. Some fibres are inserted into the urethra and vagina so that the muscle acts as a plate which is lying over the urogenital hiatus. Anal ultrasound studies have shown that the urogenital hiatus widens when the levator ani muscles relax and the supporting ligaments are left to hold the uterus and other viscera alone.[1]

The right and left puborectales unite behind the anorectal junction to form a muscular sling. Some regard them as a part of the sphincter ani externus. The levator ani muscles as well as the external anal sphincter muscle are innervated by the pudendal nerve, deriving from S2-4.

The muscles and vagina interact with the different ligaments. DeLancey describes the anatomy of vaginal vault prolapse in terms of 3 levels of support

- Level I involves the support of the upper vagina and cervix or the vaginal cuff (in a woman who has undergone total hysterectomy) by the cardinal-uterosacral ligament complex.
- Level II denotes the lateral support of the mid vagina to the arcus tendineus fascia pelvis (white line).
- Level III is represented by the fusion of tissue along the base of the urethra and the distal rectovaginal septum to the perineal body.[2]

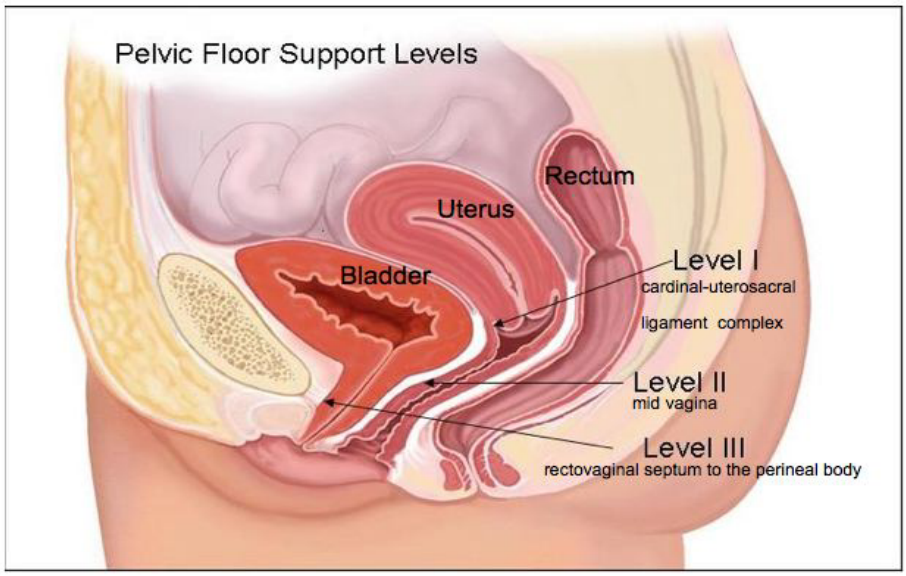

Figure 1.1 Three levels of support (from DeLancey)

The endopelvic fascia is a tissue compound overlying the entire pelvic floor and connecting the different muscle parts and forming the pelvic ligaments thus granting extra support. The fascia is mainly composed of connective tissue and smooth muscle.

Anteriorly, it forms the pubourethral ligaments arising either side of the urethra and supporting its proximal aspect. These ligaments extend anteriorly and end as

the suspensory ligaments of the clitoris. Further the pelvic fascia extends posteriorly and so connects the symphysis pubis with the ischial spine. This is the “arcus tendineus fascia pelvis” also commonly referred to as the “white line”.

The uterosacral and cardinal ligaments are important ligaments that form from fascial thickening at the posterior and lateral aspect of the uterus and the parametrium and paracolpium are fascial continuations around the uterus and vagina respectively.

1.3 Vaginal Wall

1.3.1 Anatomy and Histology of the Vagina and the Vaginal Wall

The vagina is not a support structure for the pelvic floor as such but its anatomical direction plays a role in preventing POP. It stretches at a length of around 7-8cm from the introitus vaginae posteriorly tilted upwards to the uterine cervix. It is supported and kept in its anatomical position by the paracolpium, the urethrovesical ligaments and the pelvic fascia between bladder anteriorly and rectum posteriorly.

The vagina is of dual origin during embryological development. The caudal part derives from the sinuvaginal bulb and the cranial part from the uterine canal (paramesonephric ducts) thus connecting the inner genital organs with the exterior part.[89] The lower part, is mainly but not totally, closed by a fibrous tissue ring, the hymen. The fibrous-muscular vaginal wall is only 2-4 millimetres thin and

has four layers. The mucosa is combining the epithelium and lamina propria. It consists of only 30 cell layers and is non-keratinized squamous epithelium.

The layers of the vaginal wall:

- Mucosa: stratified nonkeratinized squamous epithelial tissue with:
  - superficial stratum
  - intermediate stratum
  - parabasal stratum
  - basal stratum
  - lamina propria
- Submucosa: vascularised connective tissue
- Muscularis: smooth muscle, inner circular and outer, thicker longitudinal muscle
- Adventitia

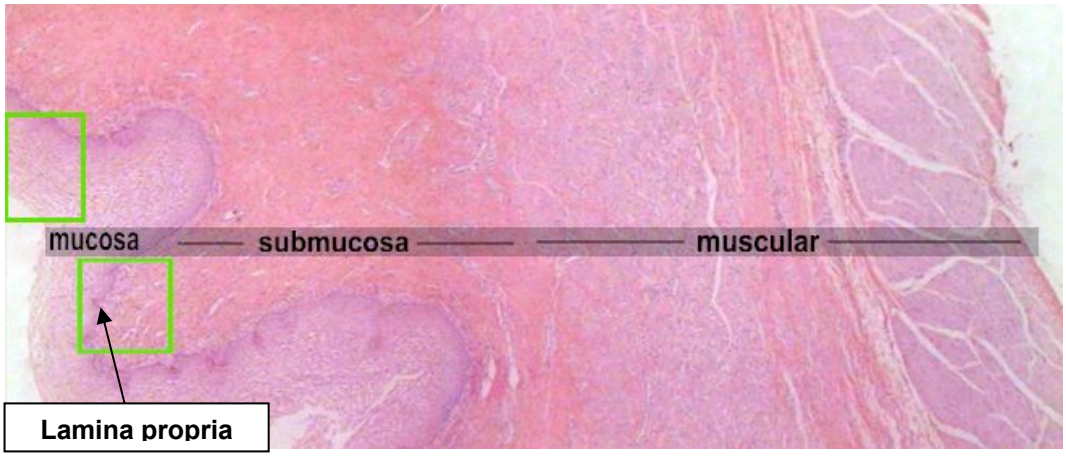

Figure 1.2 Vaginal wall histological profile

The term pelvic floor dysfunction (PFD) describes a variety of changes and disturbances in the normal anatomy and physiology of the pelvic floor components. PFD has varying end results but often with considerable and grave impacts on the quality of life and normal day to day functionality of patients. Stress incontinence and pelvic organ prolapse are types of PFD.

1.3.2 Pelvic Organ Prolapse

Pelvic organ prolapse (POP) or genitourinary prolapse is defined as the protrusion of a pelvic organ beyond its normal anatomical confines. This condition affects 15 % of the overall female population and almost 50% of parous women in later life of which 20% are symptomatic. [3] Currently surgery is the first line treatment for symptomatic prolapse. 20% of patients on the waiting lists for major gynaecological surgery are waiting for a prolapse repair. In view of distress and surgical risks, research that improves the knowledge about this condition could reduce the prevalence and improve treatment.

1.3.3 Classification and grading of Pelvic Organ Prolapse

The classification of pelvic organ prolapse is dependent on the protruding organ into the vagina. The grading describes the degree of descent into or beyond the vagina.

Classification:

Apical/Middle:	
Uterus	uterine prolapse/procidentia
Vaginal Vault	vault prolapse
Omentum and small bowel	enterocele
Anterior:	
Bladder	cystocele
Urethra	urethrocele
Posterior:	
Rectum	rectocele

Figure 1.3 Anatomical representations of different types of pelvic organ prolapse

Grading:

grade 1	protrusion of a pelvic organ into the vagina but the lowest part has not reached the hymenal ring.
grade 2	protrusion to the hymenal ring
grade 3	protrusion beyond the hymenal ring and in case of uterine prolapse: procidentia

Another grading system that attempts to establish a more specific description of prolapse is the POP-Q described in the Table below.

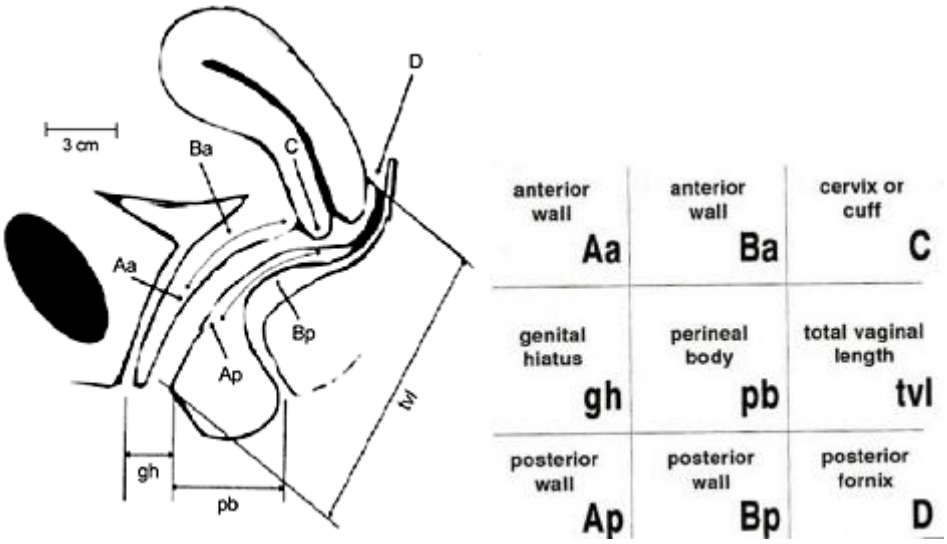

Figure 1.4 POP-Q, Pelvic Organ Prolapse Assessment Tool

Aa	point in the midline of the anterior vaginal wall 3cm proximal to the urethral external meatus
Ba	most distal position of the anterior vaginal wall from the vaginal vault to Aa
C	most distal edge of the cervix or vault
D	location of the posterior fornix
Ap	point in the midline of the posterior vaginal wall 3 cm proximal to the hymen
Bp	most distal point of posterior vaginal wall from vault or posterior fornix to Ap
GH	genital hiatus measured from the middle of the external urethral meatus to the posterior midline of the hymen
PB	perineal body measured from the posterior margin of the genital hiatus to the middle of the anus
Tvl	total vaginal length

The examination is usually performed whilst the patient is performing the Valsalva manoeuvre. Points Aa, Ab, Pa, Pb are assessed using a Sim's speculum. The anterior or posterior vaginal wall is hereby retracted to assess the opposite side for prolapse. A Cuscoe's speculum and a device marked in 1cm steps are used to measure point C and D.

The staging is similar to that of Walker and Baden:

0	no descent during straining
I	leading surface of the prolapse does not descent below 1cm above the hymenal ring
II	Leading edge of the prolapse extends from 1cm above to 1cm below the hymenal ring
III	from 1cm of the hymenal ring but without complete vaginal eversion
IV	complete eversion of vagina

The purpose of the ICS POP Quantitative Staging or Scoring System (ICS POP-Q) is a standardised form of measuring pelvic organ prolapse. It was found to be reliable and reproducible in evaluating studies.[4]

Grading systems history:

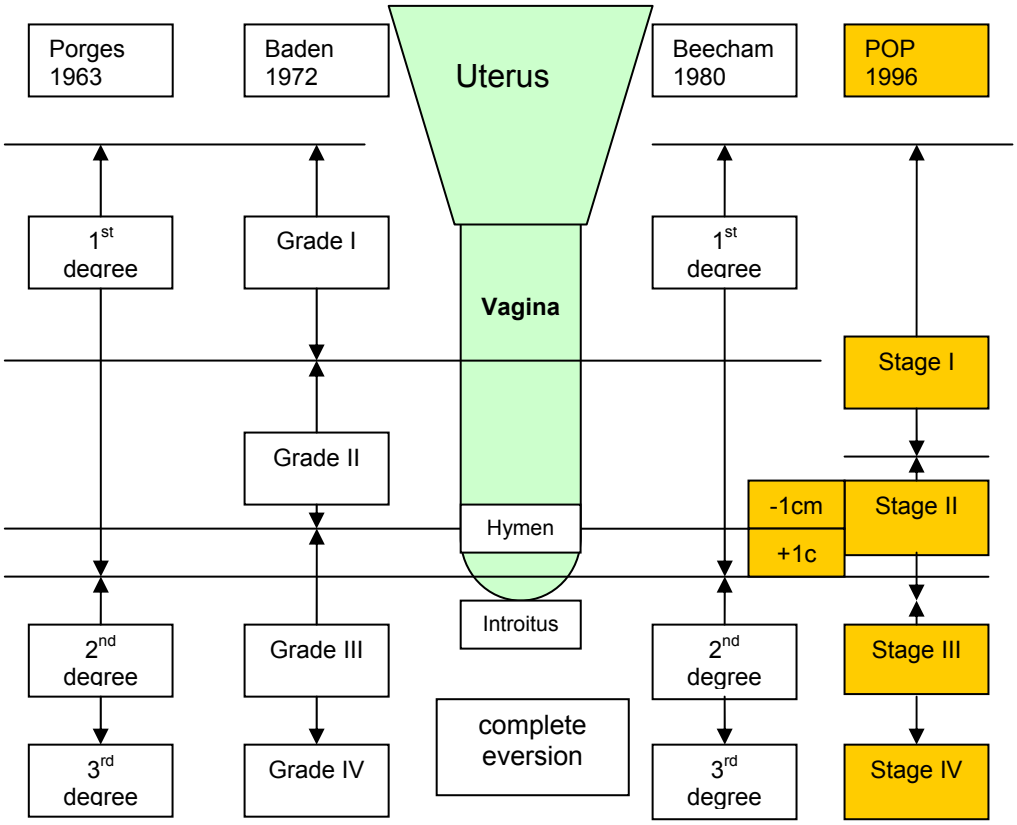

Figure 1.5 History of commonly used pelvic organ grading systems. POP is the currently used system and the system used in this study (schematic, original by Steven Swift and John Theofrastous)

1.3.4 Symptoms of Pelvic Organ Prolapse

Pelvic organ prolapse can be irritating for women and diminish their quality of life significantly. Symptoms can vary from a dragging feeling in the vagina which is often described as the feeling like “sitting on an egg” or “feeling a lump between the legs”, to significant pain abdominally or in the back. Ulcerations and infections are common complications and dyspareunia or other problems during intercourse such as urinary or faecal which not as uncommon as previously thought can lead severe impairment in functioning for patient and partner. In 60% percent of the cases either stress incontinence symptoms or urgency with urge incontinence occurs or both are present as mixed incontinence.[5]

1.3.5 Management of Pelvic Organ Prolapse

1.3.5.1 Examination

An initial detailed history should be taken and the patient assessed thoroughly including symptoms, their severity and length of time that the problem has been present. This is followed by a gentle but thorough abdominal and pelvic examination. Abdominal palpation is crucial to exclude any underlying pathology that is not related to prolapse such as pelvic masses which could be of ovarian origin or uterine fibroids. Vaginal examination is usually performed bimanually in dorsal lithotomy position but an upright position with one of the patient’s legs standing on a chair can give a better and more obvious assessment of the prolapse. Additional speculum examination, either with a Cuscoe or Sims speculum is required to assess not only the prolapse but also health of cervix and

vaginal walls. Examination with a Sims speculum should be performed in the left lateral position. A rectal examination should be performed to exclude or assess a rectocele, enterocele and to identify possible anal sphincter dysfunction.

The pelvic floor muscles need to be examined to establish strength and function. For this the patient lies on her back with the legs in parallel position and tries to apply pressure to the examiners digits. The thickness of the pelvic floor muscle diaphragm should be felt and the length of pelvic floor contraction established.

Patients with additional urinary incontinence might need urodynamic studies prior to surgery and patients with rectal or faecal problems may require a referral to colorectal specialists for anorectal manometry, transanal ultrasound scanning or defecography to exclude sphincter defects or motility problems.

There are different approaches available to improve prolapse symptoms or quality of life issues. The patient can often be offered a choice between conservative and surgical options.

1.3.5.2 Conservative Options

The least invasive treatment is the most common and most important option. Obesity, smoking or chronic cough should be treated initially. Conservative options include pelvic floor exercises. These are mentioned as a possibility but not recommended if the prolapse is larger than grade 1 or only if the patient is either not suitable for or interested in other possibilities.[6]

Other conservative options are vaginal pessaries in various shapes and sizes. The one most commonly used in the UK is the Hodge-ring-pessary. Made of flexible latex, it is available in sizes between 30 and 100mm in diameter and gives support to the anterior and posterior vaginal wall as well as any apical descent. Other pessaries such as are the Simpson shelf pessary available in size 8 to 14, or the Arabin cube pessary. (Latter is not routinely being used in the UK.) A large variety of pessaries with or without extra support of the urethra are used in other countries such as USA, Australia or Canada[7].

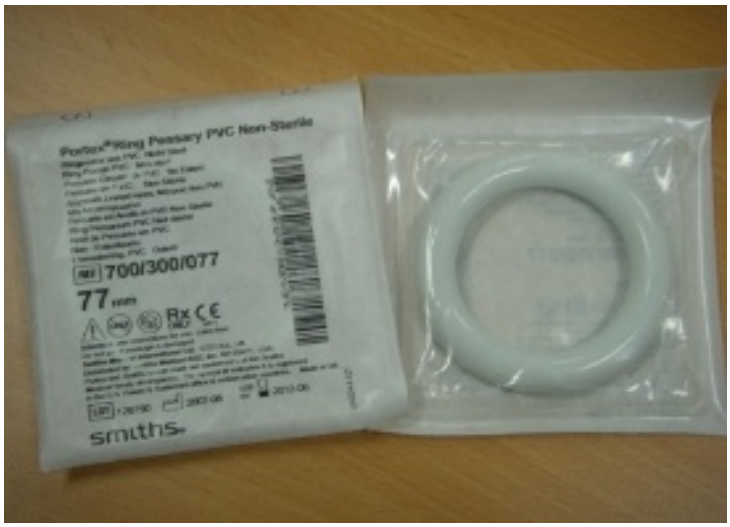

Figure 1.6 Hodge standard ring pessary, here size 77mm

Figure 1.7 Simpson shelf pessary, here size 13

1.3.5.3 Surgical Options

Since the beginning of the 20th century various surgical techniques have been described for pelvic organ prolapse. White described that for treatment of a cystocele the surgeon should reapproximate the lateral vaginal fornices to the arcus tendineus. Kelly strongly supported the plication technique and midline plication for cystocele repair. Richardson[8] published his classification of fascial defects in 1976 in which he illustrated lateral, transverse (superior), and midline defects in the pubocervical fascia. Repair was directed at the site of the specific defect. The definition of paravaginal defects was characterised as a detachment of the endopelvic fascia from the arcus tendineus fascia pelvis. These changes were found in 67% of patients with anterior vaginal prolapse. Paravaginal repair techniques were developed to repair this type of prolapse. These can be performed via the vaginal or the abdominal approach. Benson[9] for example defends the abdominal approach after he discovered that the pudendal and perineal nerve terminal motor latencies after vaginal dissection were delayed.

Anterior Compartment

Some form of the anterior colporraphy is still used as the gold standard to repair a cystocele or urethrocele. This technique can be used in combination with a sling plasty such as the tension free vaginal tape insertion when urodynamically proven stress incontinence co-exists. In later years the insertion of either organic or synthetic mesh (medium pored mesh, mainly polypropylene) which is attached to the pelvic fascia on either side of the prolapse to secure extra support to the tissue, has been used to attempt to reduce the risk of recurrence. However, they are associated with an increase in surgical complications, like mesh erosion,

fistula and dyspareunia. Raz et al [10] developed a new technique to repair large anterior vaginal wall defects such as grade IV cystourethrocele. An anterior colporrhaphy is performed to correct the central defect of the cystocele and in addition a repair of the paravaginal herniation of the bladder and bladder neck is performed by a needle suspension.

Paravaginal repair:

A paravaginal repair for the correction of a cystocele attaches the lateral vagina at the level of the arcus tendineus. A longitudinal incision of the anterior wall of the vagina is made and dissected upwards and laterally along the inferior pubic ramus, medially to the pubis and laterally to the ischial spine. Stitches are positioned in the white line, into the pubocervical fascia and into the vaginal epithelium. Further stitches are positioned posteriorly and towards the ischial spine. They are attached to the vaginal apex. The vaginal muscularis can be plicated in the midline. Redundant epithelium is excised before closure with absorbable sutures.

Abdominal and laparoscopic paravaginal repair:

Scotti et al recruited 40 women with lateral vaginal wall detachment. Paravaginal fixation to the ischial periosteum and obturator membrane was performed. The suture was passed through the arcus tendineus and obturator internus muscle, piercing the periosteum and obturator membrane after careful palpation of the ischial spine and sciatic foramen to ensure safe and anatomically correct suture placement. He found that the ischial periosteum and obturator membrane are consistently strong reattachment sites so that repair of paravaginal defects with these tissues is effective and safe.[11]

Posterior Compartment

Two different routes to repair posterior vaginal wall defect are used. The vaginal route is preferred and performed by gynaecologists meanwhile the transanal route is referred by colorectal surgeons. Here are examples for gynaecological approaches.

Posterior colporrhaphy:

Posterior colporrhaphy is performed through a midline or diamond shaped incision in the posterior vaginal wall. Interrupted sutures are set in the rectovaginal fascia and the margins of the levator ani muscles are plicated with a second layer of interrupted stitches. The vaginal mucosa is trimmed and closed with delayed absorbable sutures.

Transverse repair:

Marek et al modified a transanal technique in 1969[12]. One finger is inserted into the rectum and the margins of the rectocele demarcated. Allis clamps are placed laterally and traction applied. The mucosa is removed from side to side, superiorly and inferiorly until the rectocele is exposed. The wound is closed with a continuous lockstitch suture through the perirectal fascia. This stitch can include the superficial muscularis layer of the rectum.

Transrectal repair:

The incision in this technique is made 1 cm above the dentate line. Dissection is performed anteriorly and laterally. The rectal muscle and the rectovaginal septum in this area is plicated with interrupted sutures. Closure of the mucosal defect follows with a second layer.

Laparoscopic rectocele repair:

With the availability of minimally invasive instruments, laparoscopic techniques to effect enterocele and rectocele repair can be used. Cadeddu et al examined women that underwent a transperitoneal laparoscopic enterocele repair prior to a transvaginal rectocele or cystocele repair or laparoscopic colposuspension. The enterocele was repaired utilizing a modified Moschowitz technique. The cul-de-sac was obliterated by approximating the posterior vaginal fascia to the anterior wall of the rectum with a running suture. The group had no morbidity, an average length of stay of 3.3 days and all patients were asymptomatic with no enterocele recurrence identified during a mean follow-up of 10.5 (range 7-15) months.[13]

Fascial repair:

Denonvilliers, Uhlenhuth[14] and others demonstrated the existence of a rectovaginal septum. Richardson attributed defects in this fascia as the cause of rectoceles. He was one of the first gynaecologists to support the reapproximation of these fascial breaches to restore anatomy integrity.[15] A diamond shaped incision at the introitus is made and the vaginal mucosa reflected laterally. Whilst one finger is placed in the rectum the edges of the connective tissue are approximated. The edges are plicated with interrupted sutures and redundant skin is excised and edges sutured.

The outcome of all techniques is dependent on the pre-existing tissue. The degree of tissue damage, already present through the present prolapse, influences the possibilities to restore normal anatomy. The lower the tissue quality is, the higher is the recurrence rate after surgical repair. Mesh can be used if tissue quality is poor. It can add extra support and various types of meshes have been introduced and used for the anterior and the posterior vaginal wall. There are synthetic and organic options. A disadvantage with both groups is

increased risk of infection, erosion and dyspareunia. Therefore meshes are used for recurrent cases or elderly patients who are preferably not sexually active.

Uterine Prolapse

The method of choice for a uterine prolapse is the vaginal hysterectomy if the patient has completed her family. If the patient wishes to maintain her uterus a sacrohysteropexy can be performed where the posterior uterovaginal junction is attached to the anterior longitudinal ligament of the first or second sacral vertebra with a polypropylene mesh. A similar procedure is the sacrocolpopexy which is performed for a vaginal vault prolapse after hysterectomy. Instead of the cervix the vaginal vault is connected to the sacral periosteum with a polypropylene mesh.

Other methods are the sacrospinous or ileococcygeal fixation and a newer but more doubtful option is the infracoccygeal sacropexy, more commonly known as posterior intravaginal sling plasty (IVS). This operation involves a piece of surgical mesh attached to the top of the vagina. The mesh extends through the pelvic sidewalls and emerges from the buttock on each side. It acts to strengthen and replace the weakened uterosacral ligaments which suspend the top of the vagina in its normal anatomic position. Many other mesh kits have been used and are currently awaiting appropriate evaluation.

Prevention of prolapse is a more important than developing surgical technique as the outcome can vary significantly. It is vital, if possible, to improve the quality of pelvic floor tissue in susceptible women prior to development of pelvic organ.

1.3.6 Stress Incontinence

Stress incontinence (SI) is defined as involuntary leakage when strain is applied to the pelvic floor and urethral sphincter muscle through an increase in intra-abdominal pressure, e.g. whilst coughing, sneezing or during physical exercise. The International Continence Society (ICS) defines stress incontinence as an involuntary loss of urine which is objectively demonstrable and a social and hygienic problem". Urodynamic stress incontinence is a cystometric diagnosis defined as “the involuntary loss of urine that occurs when, in the absence of detrusor contraction, the intravesical pressure exceeds the maximum urethral pressure. [16] Accurate and detailed history taking, thorough examination and urodynamic studies confirm the diagnosis. During urodynamic assessment leakage is confirmed with absence of detrusor muscle overactivity.

Stress incontinence is caused by either urethral hypermobility occurring through deficiency of support by the surrounding tissues or genuine weakness of the urethral sphincter muscle. The currently still used classification of stress incontinence was developed by Green in 1962:

Type I	absence of the posterior urethrovesical angle without urethra hypermobility
Type II	absence of the posterior urethrovesical angle with urethra hypermobility
Type III	fixed urethra with low urethral closure pressure (<20cm H_2O)

1.3.6.1 Maintaining Continence

Urinary continence is sustained by various factors. It depends not only on urethral sphincter tone, providing urethral closure pressure, but of further importance is the position of the bladder neck. It has to be positioned intraabdominally so that increases in intra-abdominal pressure are transmitted equally to bladder and bladder neck. Through the simultaneous rise in intravesical and urethral pressure, the urethra is compressed and leakage prevented. Posterior angulation also helps to prevent leakage when a rise in intraabdominal pressure occurs. Through connection of the anterior vaginal wall, levator ani and arcus tendineous, the endopelvic fascia and the paravaginal tissue, all acting as a hammock underneath the urethra, increases in intra-abdominal pressure can be counteracted.

Further urethral closure is supported by pubococcygeus muscle contractions and the extrinsic urethral sphincter fibres. Petros and Ulmsten describe the pubococcygeus muscle as the first level of closure mechanism to achieve continence. The contraction of the anterior pubococcygeus muscle brings the vaginal hammock forward and closes the urethra. The second level, at the bladder neck, is made of uterosacral and pubourethral ligaments which support and fix the proximal urethra. With pubococcygeus muscle contractions an elongation backwards and downwards of the bladder neck is caused and the urethra is fixed. Muscular fibres at the urethrovesical junction shape the urethral internal sphincter. These are mainly circular fibres and are accountable for continence. These fibres are surrounded by striated muscle fibres, building the external urethral sphincter. With attachment of some fibres to the pubic bone, compressor urethrae, the urethrovaginal sphincter is composed. Smooth circular and longitudinal muscle fibres constrict and shorten urethral lumen and effectuate

the shortening and funneling of the urethra during micturition. These fibres are slow-twitch fibres and maintain a constant tone keeping the resting urethral closure pressure. The pelvic floor muscles (puborectalis and pubococyggeus) are the third mechanism of urethral closure. If trained they can be involved in urethral closure[17]. Thus, pelvic floor exercises are an effective tool to re-learn incontinence.[18]

Connective tissue is one of the important components of the pelvic floor in maintaining continence and structural support, together with muscular support. Pelvic fascia would be the obvious tissue to study as skeletal muscle is more sensitive than tendon to mechanical stimulus, seen by a more marked increase in the expression of TGF-beta 1, collagen I and III in response to muscle contraction in rat skeletal muscle compared to tendon [19]. Physical activity would not alter pelvic fascia as significantly. As vaginal skin can be taken as a representative of pelvic fascia and is easier to access, we decided to sample and study full thickness periurethral vaginal skin for both studies.

1.3.7 Treatment of stress incontinence

1.3.7.1 Conservative management

Conservative management should be the first line of treatment. Pelvic floor exercises and bladder retraining are known to be more effective if they are associated with changes in life style. Life style changes include weight loss, caffeine reduction, smoking cessation, balanced fluid intake, reducing physical strain on the pelvic floor and relief of constipation.

Women with high BMI show a higher prevalence of SI. Dwyer examined 368 women and demonstrated that stress incontinence was more common in obese women[20] . Bump et al assessed the weight loss in moderately obese women and demonstrated an association between weight loss and improvement in urinary incontinence symptoms[21]. Some studies suggest that smoking increases the risk of developing urinary incontinence. The more common chronic cough in smokers and the influence of smoking on connective tissue are factors that increase the risk of SI.

Pelvic floor muscle training is a very important part to re-establish stress incontinence. Additional electrical stimulation, biofeedback and usage of devices like vaginal cones can give extra support. Recent studies have shown the superiority of pelvic muscle training versus no treatment for urinary stress incontinence with a success rate of 68 to 74%[22]. Supportive devices such as vaginal cones or electrical stimulation were found to be very helpful in combination with PFE but not as treatment alone.

1.3.7.2 Medical treatment

A recent new serotonin (5-HT) and noradrenalin (NA) reuptake inhibitor, Duloxetine has been approved for the treatment of stress urinary incontinence. Duloxetine acts on the sacral spinal cord in animal studies where it increases the concentration of 5-HT and NA. Thus it increases the activity of the external urethral sphincter and prevents involuntary urinary leakage. There is no influence on micturition as the external sphincter muscle action is controlled by glutamine and not by 5-H and NA. Duloxetine in combination with pelvic floor exercises are more effective than no treatment.

1.3.7.3 Surgical treatment

Surgical options should be the next line of treatment. Stress incontinence should be proven by urodynamic testing, assessment of symptoms and the patient's health should be considered. The most appropriate surgical technique can then be chosen and applied. Below are some options summarized.

Bulking agents:

Bulking substances are injected into the urethral submucosa at the level of the bladder neck. They narrow the urethral lumen and thus prevent SI. The injections can be performed paraurethrally or transurethrally. The transurethral use allows a direct vision of location of the needle and the bulk effect. This technique is mainly used for patients with intrinsic sphincter deficiency and stable detrusor muscle with a non-mobile urethra. The first substance used as a bulking agent was polytetrafluoroethylene. Problems with this substance are migration into the surrounding tissues and granuloma formation. Another option is glutaraldehyde cross-linked bovine collagen. It degrades slowly in the tissue but studies have shown a success rate of 64 to 95%[23]. Other substances used are macroplastique and bioplastique made of silicone polymers. Further materials that are being studied are allogenic human collagen and autologous cartilage and synthetic agents such as microballoon technology, hyaluronic acid with or without microsphere technology

Needle suspension:

The success rate of this procedure is around 70%. Paraurethral tissue is connected to the rectus fascia with stainless steel wires. Long term results at five and ten years show a drop in the success rate to 43% [24]. This procedure is rarely used now.

Sling procedures:

Sling procedures are aiming to create a hammock like support suburethrally counteracting bladder neck descent. Autologous fascia lata, cadaveric fascia lata, homologus, porcine dermis and porcine intestinal submucosa have been used in addition to synthetic materials such as Mersilene, Vicryl, Silastic, Gore-tex, Prolene and Marlex. The synthetic materials are stronger but show a higher incidence of erosion and wound healing difficulties. The tension free transvaginal tape (TVT), was developed in 1994 by Ulmsten[25] As opposed to previous sling procedures, the TVT supports the mid urethra instead of the bladder neck. A longitudinal vaginal wall incision is made under the mid-urethra along with two suprapubic 0.5cm incisions. The paraurethral spaces are dissected bilaterally. The Prolene tape is introduced by using two curved needles on either side of the urethra aiming at the skin incisions via the retropubic space. It is important that no tension is applied with the sling as subsequent fibrosis of the mesh can shorten the structure and induce urinary retention. TVT was found to be superior in outcome, recovery and post operatively complications when compared to colposuspension. Long term outcome at 5 years is 80% [26] The transobturator tape (TOT) is a successful modification of the TVT. In this technique the tape is inserted from the inguinal region through the Obturator foramen into the paraurethral space. Studies showed similar outcome at 12 months post-surgery. One advantage of the TOT is reduced risk of bladder injury, compared to TVT. The main disadvantage is a small risk of permanent thigh pain.

1.4 Racial Differences in general and in Pelvic Floor Dysfunction

Previous studies have demonstrated that black African women have a significantly lower prevalence of stress incontinence even with similar risk factors such as parity or menopause.[27] Studies observing differences in other tissues such as bone and skin have shown differences in extracellular matrix protein content between black and white people.[28] Pelvic ligaments of black women with prolapse showed greater collagen content compared to white peers.[29] Hoyte showed that levator ani volume was significantly greater in the African-american women compared to white-american women. He also found that the levator-symphysis gap was smaller in the AA and that there were significant differences in bladder neck position, urethral angle, and the pubic arch angle.[30] Black women have been shown to have a higher bone and muscle mass and have a lower incidence of bone fracture and trauma to the connective tissues[31] [32]. Compared to Caucasian women, black women not only have a lower incidence of stress incontinence but also develop pelvic organ prolapse more rarely but they are more prone to detrusor over activity and with it urge incontinence and keloid formation in the skin.[33]

Other studies examined anatomical differences in stress incontinent women of black and white origin and found that black women possess a significantly greater urethral volume and a higher mean Kegel urethral closure pressure when measured with MRI.[34] Knobel et al demonstrated that the bladder neck in black women is situated more proximally and that the urethral length is greater. [35] More recent studies have showen that stress incontinent black women have higher collagen I and III content compared to white women and a significantly higher collagen XVII expression in periurethral vaginal skin.[36]

1.5 Pathophysiology and Risk Factors of PFD

The aetiology of POP is so far not completely established but appears to be multifactorial. Different mechanisms have been discussed and are widely accepted:

- Familial predisposition
- Body habitus and obesity
- Mechanical trauma to pelvic floor muscle, damage to nerve supply and injury to endopelvic fascia, parity
- Hormonal changes, Menopause
- Smoking

1.5.1 Genetics and Familial Predisposition

Studies examining epidemiological evidence suggest a genetic predisposition to prolapse and stress incontinence. This could be a result of various different gene abnormalities in expression of connective tissue proteins. Twiss et al summarized evidence suggesting a genetic basis for the development of urogenital prolapse and stress urinary incontinence. They demonstrated that abnormal expression of various structural proteins appears to be one of the possible causes. Families with an autosomal dominant pattern of transmission of urogenital prolapse with high penetrance have been identified. Further, candidate genes have been identified that appear to predispose women to urogenital prolapse and stress incontinence.[37] One of these candidate genes was examined by Nikolova et al in 2006. Their data for example suggest that a polymorphism in the promoter of the

laminin gamma1 gene (LAMC1) may increase the susceptibility to early-onset pelvic organ prolapse.[38] Miedel et al also demonstrated in a controlled study examining more than 800 women, that among other non-obstetric risk factors, the known familial history of conditions suggestive of deficient connective tissue such as varicose veins, hernias or haemorrhoids and showed an increased prevalence for positive history compared with no history. Also, family history of prolapse showed increased prevalence when compared with no history. However the link to family history could be partly due to information bias.[39] Other studies showed that daughters of incontinent mothers (urge incontinence was not significant) had a 1.3 fold increased risk of being incontinent. Female siblings had a 1.6 fold increased risk of urinary incontinence if their older sisters were incontinent[40]. A twin study examining the bladder neck mobility in monozygotic and dizygotic twins suggested a genetic contribution for up to 59% variance for bladder neck decent and thus pelvic floor dysfunction[41].

1.5.2 Body Habitus and Obesity

Whitcomb et al found that the prevalence of pelvic floor disorders in obese women increased with higher degrees of obesity.[42] These findings, supported by various similar studies, would concur with clinical experience. It is known that fat cells are target cells for oestrogen. Nilsson et al showed that ER alpha mRNA expression levels were lower in obese compared to non-obese women in both subcutaneous adipose tissue and in adipocytes. These findings support a role for oestrogen signalling via ER alpha, in control of body weight.[43]

Excessive weight does also mechanically challenge pelvic floor integrity. In combination with changes in the hormonal balance of obese women this would

furthermore compromise the strength of connective tissue and muscle. Wasserberg et al examined the changes in quality of life and symptoms of pelvic floor dysfunctions in morbidly obese women that had undergone surgical weight reduction. Significant differences in pelvic floor dysfunction symptoms were reported.[44] Kapoor et al also found in their pilot study in 2004 that morbid obesity is associated with a significant negative impact on urogenital health.[45]

1.5.3 Mechanical Trauma through Surgery to Pelvic Floor Muscle, Damage to Nerve Supply and Injury to Endopelvic Fascia, Pregnancy and Parity

Mechanical trauma, for example during child birth has been shown to contribute to the development of POP. However pregnancy alone changes connective tissue through increasing laxity and collagen degradation. Rectus fascia from pregnant women contains less collagen and has more glycosaminoglycans.[46] Bladder and urethral mobility increase in pregnancy, as well as elbow hyperextension, as early as 6-18 weeks gestation.[47] The cervix of pregnant women in the third trimester contains less collagen I than the cervix of women in the first trimester.[48] It is possible that these findings imply long term changes but so far no further studies are available.

Increased birth weight, forceps delivery and perineal trauma seem to increase the risk of pelvic organ dysfunction postpartum while vacuum delivery and the length of second stage seem not to increase its incidence. Dietz et al showed in 2006 that inferior aspects of the levator ani and fascial pelvic organ supports such as the rectovaginal septum can be injured during child birth. This is associated with pelvic organ prolapse, bowel dysfunction, and urinary incontinence. Pudendal

nerve terminal motor latency is prolonged in women after vaginal delivery and seems to be longer in multiparous women compared to primigravidae. Further, the review found that older age at the first delivery could be associated with a higher possibility of trauma.[49] This again could demonstrate that age related changes in the pelvic floor are under the influence of oestrogen and the impact of hormonal changes over time. In another study, Dietz et al confirmed the high incidence of incontinence after vaginal delivery. They showed that injury of the inferomedial aspects of the levator ani from the pelvic sidewall occurred in approximately one third of all women delivered vaginally and was associated with stress incontinence 3 months after childbirth.[50]

Recent studies experimenting with endoanal ultrasound and magnetic resonance imaging of the pelvic floor after vaginal delivery showed areas of muscle insufficiency and thinning as a direct outcome of child birth. This was shown during straining and during pelvic floor activity. [51] Nevertheless, it provides evidence of changes in relation to trauma.

When parous twins were compared in an identical twin study, it was found that performing a caesarean section had a 3 fold reduction in SUI risk relative to the siblings that delivered vaginally. 200 women were followed up between 2 to 5 months postpartum and results demonstrated that those who delivered vaginally, especially with forceps causing the most changes, showed higher increased pelvic organ mobility at valsalva manoevre than those who had any type of caesarean section.[52] Nevertheless other studies suggested that caesarean section during active labour and vaginal delivery has similar effect on the maternal pelvic support 6 weeks postpartum.[53] Pelvic floor muscle strength is found to be reduced after vaginal delivery but it is restored almost back to antepartum values in 2 months.

MRI images showed that the pelvic floor of women with vaginal prolapse had a 2.5 times increase in likelihood to have major levator ani muscle loss after vaginal birth compared to the controls.[54] Furthermore women who had episiotomies during vaginal delivery seem to complain more of dyspareunia after delivery than women who delivered vaginally without episiotomy.

1.5.4 Hormonal Changes and Menopause

The incidence of POP for women after the menopause is significantly higher compared to women before the menopause. Some studies demonstrate that a lack of oestrogen is a predisposing factor for pelvic organ dysfunctions and even that SERMs (Selective Estrogen Receptor Modulators) can induce prolapse or worsen the symptoms. However other studies also indicate that some SERMs such as raloxifene taken long-term do not appear to adversely affect urinary incontinence or prolapse. [55]

Reay Jones et al were able to show that the resilience of uterosacral ligaments decreases with vaginal delivery and further during the menopause. 85 uteri were examined and uterosacral ligament resilience measured. Correlation with uterocervical prolapse, patient age, history of vaginal delivery and menopause was shown.[56] With weak pelvic floor muscles a decrease in pelvic connective tissue resilience related to the menopause could aid progression to symptomatic pelvic organ prolapse. Previous studies and clinical experience appear to have shown that oestrogen alters the extracellular matrix of different tissues in the female pelvic and urogenital tract in stress incontinent women or women suffering from pelvic organ prolapse. Further oestrogen can improve the symptoms of prolapse and urge or stress incontinence significantly so that imminent surgery can be avoided or at least postponed.

The latest Cochrane review of 28 trials which overall reviewed 2926 women assessed the effects of oestrogens used for the treatment of urinary incontinence. It was found that oestrogen treatment can improve or cure incontinence and the evidence suggests that it is more likely with urge incontinence. There were not sufficient data to confirm reliably other aspects of oestrogen therapy such as oestrogen type, dose and route of administration.[57] These effects appear to happen through remodelling of the various components of the extracellular matrix, most importantly collagen and elastin. It would be of interest is to observe how exactly the expression of the oestrogen receptor is changed under the influence of the hormone. Ewies et al showed for example that the clearly discernible levels of expression of ER alpha, ER beta, androgen receptor (AR) and progesterone receptor (PR) in prolapsed cardinal ligaments suggest a relationship to the process of tissue stretch 'trauma', rather than an effect of the menopausal status, HRT use or cell proliferation. The use of HRT in post-menopausal women appeared to offset some of the changes observed with the prolapse. [58] It was demonstrated that oestrogen receptors were present in the vaginal wall and uterosacral ligaments and that the expression of the different oestrogen receptors were altered in menopausal women.[59]

In the present pilot study using topical oestrogen on prolapsed vaginal wall tissue, it was hypothesised that the treatment with direct oestrogen could alter the extracellular matrix and perhaps improve symptoms and quality of life of postmenopausal women with POP. It was also intended to study molecular biology changes that had not been investigated before in vaginal skin of women with POP after oestrogen treatment and to determine if such changes were progressing towards those required for improving the resilience of vaginal skin. This is the first study to examine the changes of extracellular matrix components

under the influence of topical oestrogen for a time interval and the first one to assess oestrogen receptor α expression in this setting.

1.5.5 Smoking

It is known that smoking tobacco influences the biochemical mechanisms of blood vessels and physiological functions of connective tissue throughout the entire human body. Not only the contained Nicotine, an alkaloid found in the nightshade family of plants (Solanaceae) which constitutes approximately 0.6–3.0% of dry weight of tobacco but other compounds of the most commonly used cigarettes and known as carcinogenic, contribute to cardiovascular diseases and deterioration of the quality of skin. Previous studies showed that smoking has an accelerating effect on the skin aging process.[60] There is further a direct negative effect on the elastic fibres in subcutaneous skin tissue resulting in skin laxity and reduced elasticity.[61]

Araco et al recently showed that smoking increases the risk of post-operative complications such as mesh erosions after POP repair where meshes were used to enhance surgical outcome.[62] Smoking is now acknowledged as one of the main risk factors that contribute to development of POP. This information needs to be included in the history of patients of this study and differences between smokers and non-smokers need to be statistically assessed.

1.6 Extracellular Matrix (ECM) Components of the Pelvic Floor and Vaginal Tissue

The adult ECM has the purpose to not only resist tensile forces but also to counteract compression. So fibres are required in the ECM that are able to achieve both stability and elasticity. The ECM must also provide permeability for small molecules to be able to diffuse through the tissue. The ECM contains two main components, elastic fibres and collagen. Reticular fibres are also part of the tissue but are part of the collagen group. These proteins are produced by fibroblasts but collagen can also be synthesised by chondroblasts, osteoblasts and smooth muscle cells.[63]

1.6.1 Collagen

Collagen is the most abundant protein in the adult human body making up 30% of the entire protein content.[64] There are various collagen types in existence and each of them fulfils a different task in their numerous locations, some being more elastic than others. Only the collagen types examined in this study are described in more detail.

So far nineteen types of Collagen have been identified and 12 have been studied closely. They can be categorised into seven main groups.

Group 1: Fibrilar collagen

Type I	composing fibrils in dense and areolar (loose) connective tissue of skin, tendon and bone
Type II	only in cartilage
Type III	part of reticular fibres, mainly in loose connective tissue, vessels, uterus, skin
Type V	very thin fibrils, combined with Type I and III interstitial tissue and vessels
Type XI	in cartilage, combined with Type II

Group 2: Fibril associated collagen

Type IX	surface of Type II in cartilage
Type XII	surface of Type I in dense connective tissue
Type XIV	as Type XII
Type XVI	location unknown
Type XIX	embryogenic in brain, eyes and testes

Group 3: Microfibrilar collagen

Type VI	thin filaments in dense and loose connective tissue and cartilage
Type VII	in Skin, anchor protein of the dermo-epithelial connection

Group 4: Collagens of basal lamina

Type IV	in Lamina densa of the basal lamina, part of anchor complex, contains six different alpha chains in various combinations

Group 5: Short chain collagen

VIII	in cornea (Descemet membrane) and subendothelium of vessels
X	cartilage, particularly epiphysis

Group 6: Transmembrane Collagen

XIII	between cellular junctions and cell-matrix junctions
XVII	in Hemidesmosomes

Group 7: Multiplexin Collagen

XV	in basal membrane and pericellular matrix
XVIII	unknown

1.6.1.1 Collagen I

The most common and most important collagen for tensile strength is Collagen Type I. In skin, 80% of the collagen is type I, where it is responsible for the tensile strength of the tissue. Alterations in its gene result in various forms of osteogenesis imperfecta and Ehlers-Danlos syndrome type VII which is characterized by joint hypermobility and, at the same time, is associated with an increased risk of developing pelvic organ prolapse.[65] Together with Type III, IV, V, XI it belongs to the group of fibrilar collagens, important collagens in connective tissues.

1.6.1.2 Collagen III

Fifteen per cent of the collagen content in skin is type III which is accountable for elasticity of tissues. The ratio between collagen I and III demonstrates the characteristics of the tissue. A higher ratio shows that the tissue is more tensile, a lower that the tissue is more elastic.[66] Alteration of the ratio has been found in women with stress incontinence.[137]

1.6.1.3 Collagen XVII

Collagen XVII is a collagenous transmembrane collagen. It is composed of an alpha-chain which contains an N-terminal intracellular domain, a hydrophobic transmembrane area and a larger, extracellular C-terminus. Collagen XVII is a

structural constituent of the hemidesmosomes. Hemidesmosomes arbitrate adhesion of the epidermal keratocytes and other epithelial cells to the underlying basal membranes. Collagen XVII is involved in the maintenance of the epithelial cell adhesion through multiple protein–protein interactions. This is demonstrated especially through genetic and acquired skin diseases. The lack or loss of collagen XVII function leads to diminished epidermal adhesion and skin blistering. Collagen XVII is shed from the cell surface yielding a shorter soluble form of the molecule by proteinases of the ADAMs family (ADAM-9 and ADAM-10). The functional consequences of shedding of collagen XVII are not yet clear[67], [68] It is important to give insight into the basic construct of the collagen fibres to understand the function appropriately.

1.6.1.4 Structure and molecular construction of Collagen

Collagen is mainly produced by fibroblasts of connective tissue and released into the extracellular matrix. Collagens are arranged by collagen fibrils which are between 30-70 nanometres wide. Collagen fibrils are usually composed in the shape of a triple helix, in case of collagen type I it is an assembly of two alpha1 and one alpha2 chain. These chains are arranged in the procollagen fibre which is then shortened to tropocollagen (Figure 1.8). Tropocollagens are arranged adjacent to each other overlapping the neighbouring molecule by a quarter of its length thus giving the typical electromicroscopic picture of steps (Figure 1.9).

Pictures of collagen molecules and fibrils

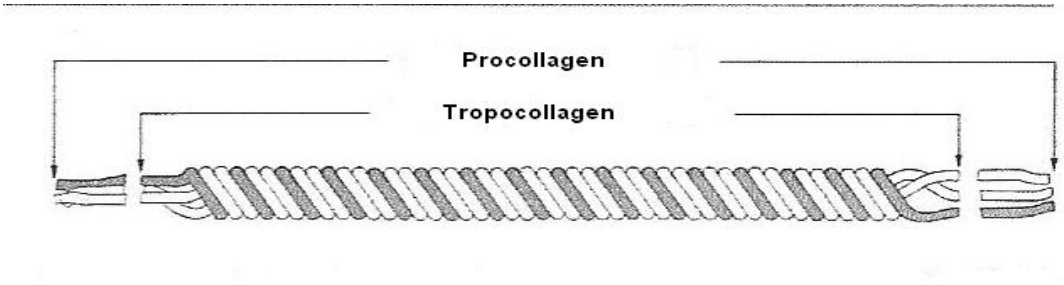

Figure 1.8 Collagen Type I molecule. Two α1-chains (white) and one α2-chain (grey) building a triple helix

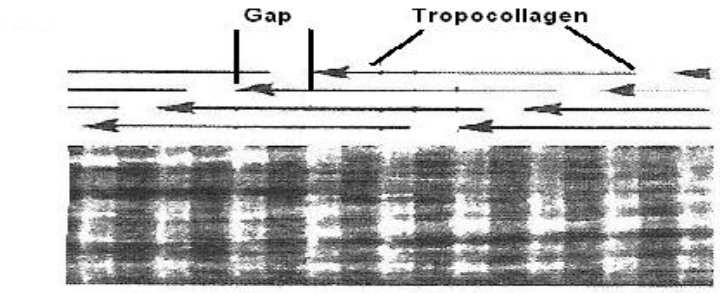

Figure 1.9 Molecular structure of collagen fibrils. tropocollagen (arrows) is overlapping the adjacent molecule by ¼. (schematic by Benninghoff)

All collagens contain a high amount of the aminoacids glycine, proline and hydroxyproline. Glycine is in each third position of the molecule and is essential for creating the structure of the triple helix. Mutations that interfere with this arrangement are responsible for various diseases.

The alpha helices of procollagen are constructed in the endoplasmatic reticulum of fibroblasts. One important step for the synthesis is the hydroxylation of proline through proline hydroxylase which requires Vitamin C as a co-factor. Thus

Vitamin C deficiency can lead to insufficient collagen synthesis and scurvy. Lysine is also hydroxylated besides proline and further oxidised to an aldehyde formation through lysyloxidase which requires copper as a co-factor. Procollagen is 10-15 nanometre longer than tropocollagen. It is spliced by procollagen peptidase removing the propeptides at the C- and N-terminals.

The lysine aldehyde groups of the final tropocollagen arrange the molecule through covalent connections that support a stable structure and stabilisation of the fibrils. These cross-links between the molecules are initially divalent dehydro-hydroxylysino-norleucine (Δ-HLNL) and hydroxylysino-keto-norleucine (HLKNL). With maturity of the tissue they are converted to more stable trivalent cross-links, histidino-hydroxylysino-norleucine (HHL) and hydroxylysylpyridniline (pyrid) respectively.[69] There is a high turnover of collagen fibres and fibrils. For example Type I collagen the half-life in gingiva of rats is 2-3 days.[70] Mechanical strain and demands are a main stimulus for synthesis and turnover.

1.6.1.5 Degradation of Collagen

The main group of enzymes breaking down collagen are the matrix metalloproteinases (MMPs). (see MMP section later)

1.6.2 Elastin Fibres

Elastin is another central protein in the connective tissue and extracellular matrix of the human body. Its function is mainly to provide elasticity in tissues where tensile strength is not solely required and would challenge function. It is an amorphous protein, mainly composed of the amino acids glycine, valine, alanine and proline. In contrast to collagen it lacks hydroxyproline and hydroxylisine. It is found in oxytalan, elaunin and elastic fibres which are part of the elastic network.[71]

The Elastin gene lies on the long arm of chromosome 7 and codes the precursor molecule of elastin, tropoelastin. The genome is largely decoded and it was found that functionally distinct hydrophobic and cross-linking domains of the protein are segregated into separate exons throughout the gene. All exons are multiples of three nucleotides, and exon-intron borders always split codons in the same way which permits cassette-like alternative splicing. It is further known that transcription is initiated at multiple sites in the gene.[72] Extensive homology of amino acid sequence exists among the mammalian species and there is in addition strong conservation of nucleotide sequences in the 3' untranslated region of the gene. Variations in the structure of mRNAs resulting from alternative splicing could explain the existence of the multiple forms of tropoelastin observed electrophoretically in several species. Different kinds of splicing patterns could occur in human populations and may contribute to aging and pathological situations in the cardiovascular and pulmonary systems.[73]

Tropoelastin is rich in lysine and contrary to elastin it is soluble. It is deposited in a net of fibrillin-rich microfibrils.[74] In a further step it is oxidized by lysyl oxidase to

produce insoluble, durable cross-linked arrays. Mature elastic fibres are a composition of a microfibrillar covering and an inner centre of amorphous crosslinked elastin. The distribution of microfibrils in dynamic elastic tissues such as blood vessels, lung, ligaments and skin is essential for the tissue function.[75] The description of the native relaxed state of the protein in terms of trans-conformational balance between extended and folded structures was previously proposed [76] and recent studies support this view. The same polypeptide sequences have been analysed for their ability to self-assemble. The majority of them were able to adopt more or less organized structures. On the whole, the results obtained gave significant insight to the roles played by specific polypeptide sequences in self-assembly and possibly in elasticity.[77]

Fibrillin-1 mutations cause Marfan syndrome, which is associated with cardiovascular, ocular (ectopia lentis) and skeletal defects. Fibrillin-2 mutations cause congenital contractural arachnodactyly (CCA) with overlapping skeletal and ocular symptoms and elastin mutations cause Williams syndrome, supravalvular stenosis (SVAS) [78] and cutis laxa.[79]

Elastic fibres are designed to maintain elastic function for a lifetime.[80] But various enzymes, matrix metalloproteinases and serine proteases, are able to cleave elastic fibre molecules.[81] The degradation is part of the physiological aging of human tissues with subsequent loss of elasticity and flexibility. It plays an important role for the incidence of degenerative changes in sun damaged skin[82], the occurrence of emphysema or aortic aneuryism [83] and also pelvic floor dysfunction.

Barros et al examined human cervical interspinous ligaments and showed that aging induces structural changes to elastic and collagen fibres in this structure.

An age-related progressive increase in collagen and mature and elaunin elastic fibres was reported but elastic fibres showed structural degenerative changes with aging. Oxytalan elastic fibres were decreased.[84] This physiological process is confirmed by many studies. It seems though that elastin and collagen degradation can be influenced or altered by hormones once the process has been initiated through strain, trauma or stretching as would be the case in pelvic organ prolapse or stress incontinence.

1.6.3 Matrix Metallo Proteinases (MMP)

Matrix Metalloproteinases (MMPs) are zinc-dependent endopeptidases. They are catalysts of proteolytic events in normal as well as in pathological tissue. There are two main groups, gelatinases and proteinases which can further be broken down into subgroups. MMPs are important for cell migration, bone elongation, wound healing, angiogenesis[85], and many more tasks in the extracellular matrix of various different connective tissue structures from skin to bone. Pathological processes are tumour growth and migration, fibrosis, arthritis, lupus, scleroderma, cirrhosis, multiple sclerosis, aortic aneurysms, infertility, and many more diseases. They are involved in many processes, including tissue remodelling and also modification or release of biological factors. Substrate specificity for the MMPs is not yet entirely known. Known substrates include most of the extracellular matrix components (e.g. fibronectin, vitronectin, laminin and tenascin). Similary, proteinase inhibitors such as a_1-proteinase inhibitor, antithrombin-III and a_2-macroglobulin are selectively cleaved by MMPs as are growth factors such as IL-1a and pro-TNF-a, IGF binding protein-3 and IGFBP-5.[86]

1.6.3.1 Gelatinases

The gelatinases are MMPs number 2 and 9. They cleave mainly Type IV collagen and gelatine and can be distinguished by different molecular structures at the catalytic domain. The gelatine binding region is positioned close to the zinc binding area and forms a separate folding unit which does not interfere with the catalytic domain. The most commonly used substrate to study MMP2 and 9 activities is gelatine (heat denatured collagen).

1.6.3.2 Collagenases

All known collagens (Types I - XIV) are substrates of different MMPs. The collagenases are capable of degrading triple-helical fibrillar collagens into distinctive 3/4 and 1/4 fragments. Collagens are the major components of bone and cartilage, and MMPs are the only known enzymes in mammals that are able to degrade them. Collagenases are MMP 1, 8, 9, and 13. Type I collagen has been used as a substrate for some MMPs in research settings. It is cleaved at the same site on all three strands, releasing ¼ and ¾ length fragments. MMP-1 cleaves intact triple helical collagen efficiently, but does not work well on other substrates. MMP-3 and MMP-7, cleave a broader range of substrates but for most of the MMPs, the substrate specificity in vivo is so far ill defined.

1.6.3.3 MMP2

MMP2 is described by the National Library of Medicine - Medical Subject Headings (MeSH) as a secreted endopeptidase. It has homology with interstitial collagenase but possesses an additional domain which is similar to fibronectin. It is 72-kDa in size and belongs to the group of gelatinases. MMP2 particularly degrades type IV collagen[87] but also cleaves collagens that are previously processed by other MMP. (e.g. MMP1) Mutations in its gene have been associated with Winchester syndrome which is a disease featuring symptoms of pathologic changes consisting of dwarfism (resulting from disturbances of the skeletal-articular system), corneal opacities, coarsening of facial features, leathery skin, and hypertrichosis.[88]

1.6.4 Other Proteins of the Extracelluar Matrix

1.6.4.1 Proteoglycans

The characteristic of proteoglycans which also gives this family of proteins their name is their structure. They are composed of a core protein with one or multiple glycosaminoglycan (GAG) side chains. These glycosaminoglycan chains can contain different carbohydrate complexes such as chondroitin or dermatan sulphate, heparin sulphate or keratan sulphate. The protein core remains similar in all groups. Proteoglycans are an important component of extracellular matrix. They form complexes with other proteoglycans, hyaluronate (Aggrecan and Versican) and proteins such as collagen (e.g. Fibromodulin, Decorin, Biglycan) or Laminin and Fibronectin (Perlecan).[89] They maintain hydration through binding

cations (sodium, potassium and calcium) and water and also regulate the movement of molecules around them. They further influence adhesion, migration and proliferation of cells.[90], [91]

1.6.4.2 Glycoproteins

Opposite to proteoglycans, glycoproteins contain only short carbohydrate chains that do not contain sulphur, glucornic or idurinic acid. They function as anchoring molecules of cells in the extracellular matrix. *Fibronectin* is mainly found in its dimeric form in blood serum or on cell surfaces in the ECM. It provides the connection of cells to collagen in tissues that do not possess a basal lamina and also binds to integrins. Mouse experiments have shown that knocking out the genetic information is lethal.

Vitronectin is similar to fibronectin but in addition is found to bind to elastic fibres. It is further a component of the coagulation system in serum. *Laminins* are constructed by three protein chains (α, β, γ) which can assemble to compose 11 types of laminin molecules. Laminins polymerise to form a network and so give stability. Several types can adhere to collagen type IV through nidogen proteins and hence attach to the lamina densa. Glycoproteins are essential for various developmental and differential processes. Genetic abnormalities lead to significant diseases as junctional epidermolysis bullosa or severe muscle dystrophy.

1.7 Changes of the Pelvic Floor and Vaginal Skin in Pelvic Floor Dysfunction

Many previous studies were able to show changes in the tissue of the pelvic floor or vaginal skin of stress incontinent women or women with POP. Changes in collagen remodelling, elastin turnover or expression of other extracellular matrix proteins were discussed and investigated. Alperin et al showed in a summary from 2006 that the vagina and its support tissues actively remodel in response to different environmental stimuli. Not only the synthesis of those structural proteins, but also the balance between the activity of the major proteolytic enzymes that degrade them and the inhibitors of proteolysis are important for remodelling tissue to maintain normal function.[92] Bakas et al assessed the procollagen production for collagen I and III in sixty eight women with genuine stress incontinence and compared these to controls. They found that the quantity of collagen type I and III was significantly reduced in patients with stress incontinence. The possible cause for the reduction in the amount of collagen in women with GSI could be attributed to either a disturbance in the translation of mRNA to protein (collagen) or increased catabolism of collagen by its collagenase.[93]

Chen et al showed that stress incontinent women demonstrated an increase in MMP-1 mRNA expression and a decrease in the inhibitor TIMP-1 mRNA expression. These findings are consistent with increased collagen breakdown.[94] However it was also shown that there was no difference in TIMP-2, TIMP-3, MMP-2 or MMP-9 mRNA expression between stress incontinent women and controls. Chen et al examined further, in different studies, the effect of oestrogen on vaginal tissue fibroblasts and compared cells from incontinent to cells from continent women. The concentrations on metalloproteinase and tissue inhibitors

of metalloproteinase protein expressions in cultured pelvic fibroblasts were measured. The study showed that periurethral vaginal tissue inhibitors of metalloproteinase (TIMP) expression from fibroblasts of continent women significantly increased with increasing oestradiol concentrations (0-100 pg/mL, P<.05) differing to fibroblasts of incontinent women in which no such effect could be demonstrated. Neither group showed a change in metalloproteinase production in response to varying oestrogen levels. These results suggest oestrogen influences collagen degradation in continent women by increasing TIMP production. Also it appears to have a reduced inhibitory effect on collagenolysis in women with stress urinary incontinence.[95] A recent study by Karam et al examined elastin expression and fibre width in the anterior vaginal wall of postmenopausal women with and without prolapse. They found that elastin expression and fibre width were significantly lower compared to controls of a similar age without prolapse. No significant differences were detected when examining smoking habits, parity, HRT use and BMI.[96] In another recent study Söderberg et al examined vaginal skin from 15 women with POP and measured collagen I, collagen III, fibrillin-1, fibulin-5, decorin, lumican and fibromodulin (leucine-rich repeat proteoglycans {SLRPs}) RNA expression. There was substantially altered gene signal for production of SLRPs, which are regulators of collagen and elastic fibre assembly. They found that decorin and lumican mRNA expression were increased 16 and 8 fold respectively in premenopausal women with POP compared to controls. Fibromodulin and fibulin expressions were significantly lower compared to controls.[97]

1.8 Oestrogen Receptors and Metabolism

Oestrogens belong to the group of steroid hormones. They are the primary female sex hormones. Like all steroid hormones they derive from cholesterol and classically readily diffuse across the cell membrane inside the cell where they interact with oestrogen receptors.[98]

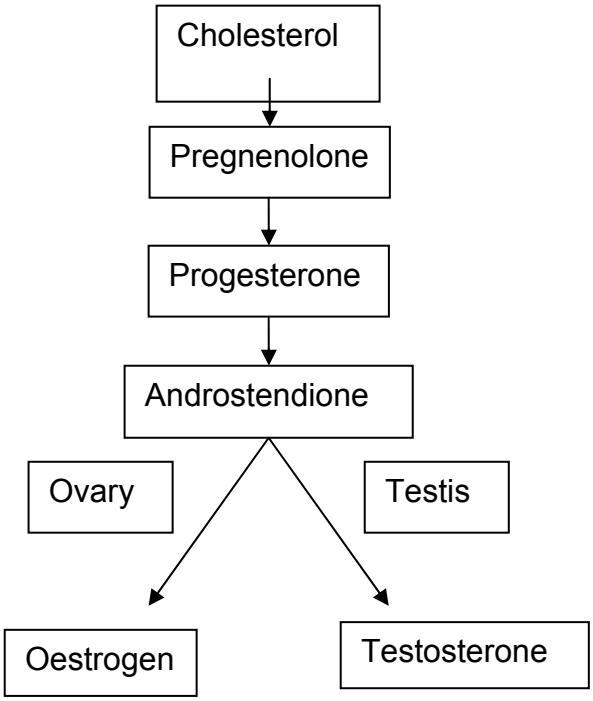

Figure 1.10 schematic Development of Oestrogen and Testosterone

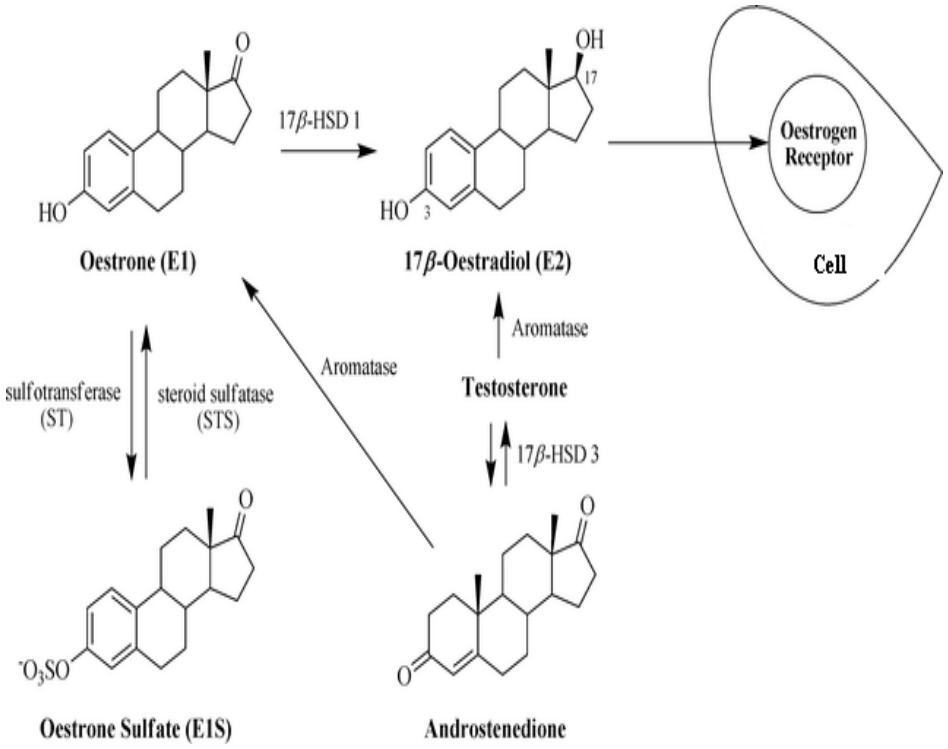

Figure 1.11 Pathway of Interaction of Oestradiol and Oestrone

The natural oestrogens are 17β-oestradiol $_{(E2)}$, oestrone$_{(E1)}$ and oestriol$_{(E3)}$. They are C_{18}- steroids which do not have an angular methyl group attached to the 10 position or a Δ^4–3- keto configuration in the A ring. Oestrogens in the female human are mainly secreted from ovarian granulosa cells of the follicles, the corpus luteum and placental tissue in pregnancy. The most important human oestrogen is 17β-oestradiol. It is converted from testosterone through the enzyme aromatase. The same enzyme converts androstendione, another C19 steroid hormone, into oestrone.

2 percent of secreted oestradiol remains free in the blood stream while the rest is bound to albumin (60%) and gonadal steroid binding globulin also known as sex hormone binding globulin (38%). All oestrogens are degraded via a liver pathway and eventually secreted in the urine as glucuronide and sulphate conjugates. Some is reabsorbed as part of the enterohepatic circulation.

Oestrogen has a distinct influence on the skin. Some studies have been able to demonstrate that oestrogen can prevent skin ageing to a certain extent. It increases the water holding capacity, enhances epidermal barrier function and shows a decrease of the alteration of skin elasticity found with time[99]. Related studies showed that postmenopausal abdominal skin areas that received hormonal treatment with application of Estrogel cream 1.5 mg/day percutaneously for 1 yr, showed significantly increased collagen content ($p < 0.001$). There was a strong correlation between the change in skin collagen content and the original skin collagen content. This indicated that the change in collagen content in response to oestrogen therapy is dependent on the original level. There is no further increase once an 'optimum' skin collagen level has been reached. [100] A national multicenter cohort study performed by Dunn, LB et al 1997 including 3875 postmenopausal women demonstrated that oestrogen use prevents dry skin and skin wrinkling, thus extending the potential benefits of postmenopausal oestrogen therapy to include protection against selected age- and menopause-associated dermatologic conditions[101].

Another study was able to show that locally applied oestrogens on abdominal skin of menopausal women enhanced the structure and content of elastic fibres compared to controls.[102] No significant changes were observed in the epidermal cell size, mitotic activity, dermal vascularization or inflammatory infiltrate between the specimens taken before and after the treatment or between the treatment groups. But not only does oestrogen affect the healthy skin and influences skin changes of physiological ageing it also influences healing capacity and so could be of importance in the direct post-operative phase after a prolapse repair.

Ashcroft et al studied the influence of oestrogens on scar healing and inflammatory effects on the cutis and found that topical oestrogens increase the

extent of wound healing compared to placebo, with a decrease in wound size, increased collagen levels and increased fibronectin levels. Also oestrogen enhanced the strength of the wound tissue after 80 days. A decrease in wound elastase levels secondary to reduced neutrophil numbers, and decreased fibronectin degradation was found as well. It appeared that oestrogen directly inhibited neutrophil chemotaxis and altered expression of neutrophil adhesion molecules.[103] Delays in wound healing could be significantly diminished by topical oestrogen. The healing capacity of non-keratinized epithelium is larger and the healing process faster compared to keratinized skin. Also, non-keratinized skin is able to absorb oestrogen to a bigger extend as barrier components are not as extensive as in epidermis. The effect of topical oestrogen could therefore even be enhanced in vaginal tissue that is dealing with scarring.

Previous studies looking more specifically into changes in female pelvic fascia and vaginal skin tissue have shown that oestrogens have considerable influence on the cardinal ligaments and vaginal skin. Pubocervical fascia fibroblasts showed increased proliferation capacity after 17β-oestradiol treatment when compared to skin fibroblasts.[104] Philips et al demonstrated that vaginal skin resembles endopelvic fascia[105] demonstrating a correlation in expression of pro-MMP-2, activated MMP-2, MMP-9 and TIMP-2 in vaginal tissue with tissue in the uterosacral ligaments. These are specific markers of collagen metabolism in the vaginal and uterosacral tissues and it suggests that vaginal tissue reflects changes occurring in the endopelvic fascia. The changes, which were more pronounced in vaginal tissue, may be a result of the prolapse rather than cause.

1.8.1 Oestrogen Receptors and Distribution

Oestrogen receptors are nuclear receptors (NR) and are part of a superfamily of ligand activated transcription factors that modulate specific gene expression. There are more than 100 nuclear receptors identified. They are categorized into steroid receptor subfamilies:

Class I NR -classically defined as ligand dependent and will homodimerize, Class II NR -classically defined as ligand independent with potential for both homo- and heterodimerization. The oestrogen receptor (ER) is classified as a Class I nuclear receptor. It is only activated through interaction with oestrogen and oestrogen like molecules. Classically, in the absence of oestrogen, ER is embedded within the nuclei of target cells and maintained in an inactive state. Binding of oestrogen or similar ligand induces a conformational change of the ER. Changed like this, the ER undergoes homodimerization and shows high-affinity to specific sites on DNA- oestrogen response elements (EREs). Once the oestrogen-receptor complex is bound to DNA, it regulates the expression of oestrogen-responsive genes.

More recently the oestrogen receptor has been found also to have non-genomic actions and can be membrane associated and linked to signal transduction mechanisms. Some of the rapid effects of oestrogens that are too fast to be by genomic action are attributed to this. Non-genomic actions, which are initiated at the cell membrane, also appear to impact transcription. There is good evidence that the membrane-limited actions of hormones such as oestrogens, involve the rapid activation of kinases and the release of calcium. These (non-genomic) effects have been attributed in some cases to receptors different to those mediating gene transcriptions but most evidence indicate that they result from the

activity of a population of nuclear receptor molecules acting outside the cell nucleus.

Studies on oestrogen and vitamin D receptors suggest that non-genomic and genomic effects may integrate in a unique mode of action of nuclear receptor ligands, in which the non-genomic effects compose signalling pathways required for the effects at the genome level.[106] Prior to this Vasudevan et al showed in 2008 that membrane actions of oestrogens, which activate rapid signalling cascades, can also potentiate nuclear transcription in both the central nervous system and in non-neuronal cell lines. These signalling cascades may occur in parallel or in series but subsequently, converge at the modification of transcriptionally relevant molecules such as nuclear receptors and/or coactivators.[107]

1.8.2 Two Isoforms of ER

Initially it was believed that oestrogen only binds to a single receptor, now referred to as ER-α. But in recent years a second oestrogen receptor was identified and named ER-β. The two receptors share common structural and functional domains. They bind oestrogen similarly and cause a similar response once activated. But there are differences not only deriving from structure and transcriptional activities but most importantly for this study, in tissue distribution.

ERα (NCBI-OMIM) is mapped to the long arm of chromosome 6. It contains 595 amino acids with an N-terminal modulating domain, central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD).

ERβ (NCBI-OMIM) is mapped to band q22-24 of chromosome 14. It is shorter than ERα with 530 amino acids.

The functional domains are the same but it is short of a portion in the C-terminal domain which is important for the effects for certain anti-oestrogens. The regions of highest homology between the two receptors are the DBD (>95%) and the LBD (>55%)[108].

1.8.3 Mechanisms of ER Signaling

It is now known that ERα and ERβ not only homodimerize but can also heterodimerize. In addition, new mechanisms for ER activation have been delineated which adds to the complexity of oestrogen receptor signal transduction. Hormone dependent phosphorylation is suspected to be an important aspect of ER binding to specific response elements. Oestrogen receptor subtypes can regulate transcription through AP-1 response elements through direct interaction with AP-1 transcription factors c-fos and c-jun. Cross talk between different signaling pathways can also induce ER activation. Receptor tyrosine kinases and IGF receptors can indirectly activate ER through direct phosphorylation of specific residues. Conformational changes in ER structure, determined by specific ligand binding, expose different amino acids that are critical for binding to protein factors involved in targeted transcriptional activation.

Finally, ER alone does not direct gene expression. Cellular pools of co-regulatory proteins play an integral role as signaling intermediates in enhancing or repressing transcriptional activity. Generally, co-activators have intrinsic histone acetylation activity associated with increased transcriptional activity and co-

repressors possess deacetylase activity which represses gene activity by keeping chromatin in a more condensed state. [109], [110]

In the present study, presence of ERα and any change in expression in response to oestrogen was of interest. For it was previously demonstrated that there is a link between changes in oestrogen receptors in cardinal ligaments of prolapsed uteri and that of vaginal tissue[105]. A.A. Ewies undertook a study to evaluate the differential expression of gonadal steroid receptors in human cardinal ligaments of prolapsed uteri and compared it with non-prolapsed controls. The use of HRT was significantly associated with low androgen receptor and high progestogen receptor expression. However, increased expression of ERα and ERβ in the prolapsed cardinal ligaments appeared to happen because of tissue trauma after considerable stretching, rather than an effect of the menopausal status, HRT use or cell proliferation. Importantly, the use of HRT in post-menopausal women appeared to counterbalance some of the changes observed within the prolapsed tissue. [58] Mokrzycki ML et al found the presence of oestrogen receptors in the cardinal ligaments and hypothesised that an increase in steroid receptors could improve pelvic floor support.[111] Ulmsten suggested that the existence and relevance of ER alpha receptors in both the pelvic floor muscles and the round ligament suggested that this could be a scientific basis for a possible beneficial effect of oestrogen therapy and a complement to other therapies, in prolapse and urinary incontinence.[112], [113] Zhu L et al established that in the levator ani and connective tissue of postmenopausal women with POP the ER alpha content was significantly higher than in controls[114], while Copas et al showed that there was a significant increase in ER alpha expression in levator ani fascia of symptomatic patients without HRT when compared to asymptomatic age-matched women. Further, he demonstrated that ER alpha expression was significantly lower in postmenopausal symptomatic women receiving long-term oestrogen replacement

compared to matched women without HRT. Long-term oestrogenisation decreased significantly ER alpha expression.[115]

Different findings could be explained by small numbers in these studies not offering enough data and the lack of a useful Meta-analysis. Laborda at al showed in 2004, that the tissue sampling site of prolapsed vaginal tissue is markedly important. Stretched vaginal tissue showed different expression and content of extracellular matrix proteins compared to tissue that was involved in the prolapse but not stretched. Stretched tissue appeared not to be able to change or process the influence from topical hormones and therefore was not suitable for analysis.[36]

Prolapsed vaginal wall tissue has so far not been examined to show changes in expression or content of ERα. Fu. X and Ulmsten in 2003 did not look at prolapsed vaginal wall tissue but tissue of stress incontinent postmenopausal women ERα was detected in vaginal epithelial, stromal and smooth muscle cells. It was significantly more frequent in stress incontinent women compared with controls. ERα was not observed in vaginal blood vessels while ERβ was detected in epithelial and vascular smooth muscle cells of the vagina. The expression of ERα, but not of ERβ, in menopausal stress incontinent women was regulated by oestrogen or progestin replacement therapy [116].

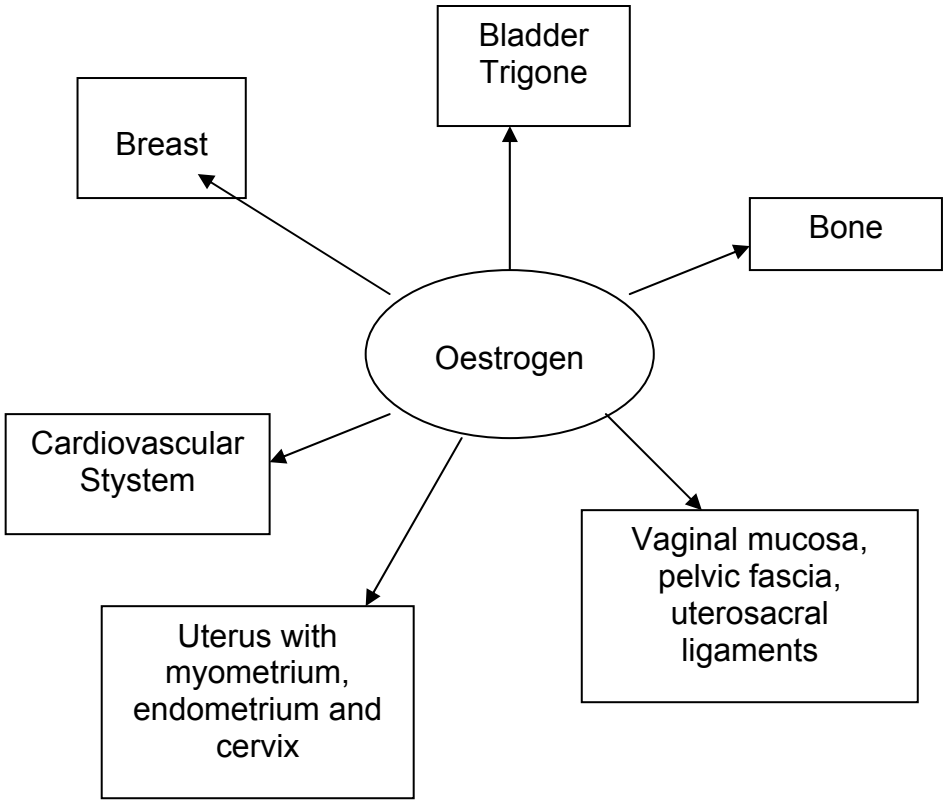

Figure 1.12 Sites of action of oestrogen and oestrogen receptors

1.8.4 Selective Estrogen Receptor Modulators (SERMS)

Selective Estrogen Receptor Modulators (SERMs) are known for their beneficial effect on oestrogen sensitive tissues and are used widely in different specialities such as Orthopaedics, Urology, Breast Surgery and Gynaecology. Many are commonly used for medical treatment of e.g. osteoporosis, breast or other gynaecological cancer and other various gynaecological disorders. SERMs include a relatively large number of compounds, each with different profiles of oestrogenic or anti-oestrogenic actions on the genital tract so they are partial or pure receptor agonists or antagonists. Their action can be different in various tissues, and therefore they have the potential to selectively inhibit or stimulate oestrogen-like action differently in different tissues.

SERMs, which inhibit breast and prostate response to oestrogens while providing oestrogen-like stimulation of bone, brain, and potentially the cardiovascular system, are already available. But so far there are no SERMs that suppress hot flushes in postmenopausal women without stimulating uterine growth. SERMs that do not stimulate mammary alveolar proliferation could be used for the treatment of endometriosis.[117]

Animal studies examined the influence of SERMS in comparison with oestrogen on variant tissues and cells. It was found that oestrogen regulated also different genes compared to SERMs, especially those genes associated with catalysis and metabolism. Genes associated with matrix integrity were also differently regulated by oestrogen and SERM, for example raloxifene. Oestradiol and lasofoxifene elevated MMP2 activity in rat uterus to twice that of raloxifene. Levormeloxifene stimulated MMP2 up to 12x that of raloxifene. There are significant differences between oestradiol and raloxifene in influencing uterine regulation of genes and proteins associated with matrix integrity. This may be a potential key difference between the actions of SERMs in the uterus of postmenopausal women.[118] These findings could also be important for pelvic floor components where SERMs and oestradiol act at similar sites and influence the extracellular matrix but different SERMS might enhance or alleviate pelvic floor dysfunction.

Recent studies looked more closely at human skin fibroblasts from post-menopausal women and studied effects of oestradiol and raloxifene on collagen biosynthesis. These studies showed that both, oestradiol and raloxifene increased mRNA expression of ERα compared to controls. Raloxifene had a greater effect on collagen biosynthesis than oestradiol and was found to inhibit

expression of MMP-9.[119] These findings are in contrast to other studies such as that of Ewies et al and thus further investigations are necessary to establish the action and effect of SERM versus oestradiol in human skin, vaginal skin and other pelvic floor components. Further knowledge could establish the therapeutic or preventative use of SERM for pelvic floor dysfunction.

1.8.4.1 Review of Oestradiol and different SERM Action on various Tissues as known so far:

Oestradiol

Uterus:

proliferation of endometrium

Extracellular matrix:

decreases MMP2, MMP9 and TIMP expression

increases MMP7 expression

Skin:

lowers pH and increases permeability

Breast:

induces cell proliferation

Bone:

increases bone density

Raloxifene

Uterus:

neutral or mild anti-oestrogenic effect on endometrium

Extracellular matrix:

MMP2 and MMP7 activity increased

no effect on MMP9

Vessels:

Induction of NO enhanced re-endotheliasation

risk of thromboembolism

Tamoxifen

Uterus:

increased growth of fibroids, increased cell proliferation on endometrium (cave – endometrial cancer)

Extracellular matrix:

no known effect on MMP9

Vessels:

increased NO enhanced re-endotheliasation

Ovary:

increased cyst formation on ovary

Breast:

acting cytotoxic and antioestrogenic

Bone:

inhibiting osteoclasts

Levormeloxifene

Uterus:

increased endometrial proliferation

Extracellular matrix:

increasing MMP2 activity 12x more than Raloxifene

Bone:

decreased bone turn over

1.8.5 SERM Effect on Pelvic Floor

It was shown that the incidence of POP during the use of the SERM levormeloxifene and idoxifene, rose 30 times higher than in controls. Pelvic organ prolapse was reported as an adverse event associated with both drugs.[120] Albertazzi et al compared different studies of the different effects of SERMs on stress incontinence and pelvic organ prolapse. The aim of this review was to systematically evaluate available data from randomized, controlled studies. Both Tamoxifen and Raloxifene seemed to increase the incidence of pelvic floor prolapse in one trial while Raloxifene did not appear to increase the incidence of urinary incontinence. Levormeloxifene and Idoxifene though were noted to increase uterine prolapse and incontinence during phase III trials. Ewies et al found that the application of cell stretch damages the F actin configuration within the cells, but impressively levormeloxifene increases the frequency and severity of these abnormalities.[121] Vardy et al were also able to show that neither raloxifene nor tamoxifen improved cytohormonal effects in the vagina or urethra compared with conjugated equine oestrogen. In addition raloxifene and tamoxifen appeared to show worsening prolapse compared with conjugated equine oestrogen and placebo. [122]

On the other hand, in a study by Parsons A et al, it was observed that either the topical use of oestradiol containing cream or nonhormonal moisturiser in women suffering with vaginal atrophy could be used in combination with raloxifene. There was no lesser effect of oestradiol when given together with raloxifene. Oestradiol containing cream improved the signs of atrophy but not the symptoms compared to nonhormonal cream in combination with raloxifene.[123]

1.9 Vagifem® and Other Pharmacological Oestrogens

Vagifem® is a vaginal oestrogen pessary which was to be used in the present study to demonstrate the effects of oestrogen on prolapsed vaginal tissue. Vagifem contains 25micrograms of 17β-oestradiol per tablet and is an easy to apply system. It is licensed in the UK to treat atrophic vaginitis for postmenopausal women. Like other topical oestrogen supplements it was thought to be useful in improving symptoms of other urogenital problems like POP or stress incontinence. The recommended dose in the BNF is 25 micrograms (1 pessary tablet) daily for fourteen days and then 25micrograms twice weekly for three months. The latter treatment can be increased to six months if required. Vagifem was used for this study as it was found to be used with a greater compliance compared to other topical oestrogens as creams or other pessaries. Other than systemic HRT preparations the side effect profile for Vagifem® is low but effects on the endometrium with long term treatment are uncertain.

Studies assessing the risk of malignancy during the usage of Vagifem® or other low potency vaginal oestrogens, showed though that vaginal oestrogen had no significant increased relative risk of endometrial neoplasia or atypical hyperplasia,

which is a premalignant or early malignant lesion.[124] However there were no details on the time frame in which the oestrogens were administered. The difference in action between orally taken oestrogens and vaginally applied oestrogens is found in the absorption and transportation of the hormones. Orally applied oestrogen is conjugated proficiently in the liver[125] but it does not bind well to gonadal steroid hormone binding globulins and therefore most of the serum oestrogen is in its active form. With oral application on a daily base the possibility of accumulation in the blood stream is high and so reaction of endometrial cells probable. Absorption of vaginal oestrogen, though it appears to be absorbed easier initially, decreases over the time with increasing thickness of the vaginal wall due to its effects. [126] Further, it was shown in this study by Nilsson et al that serum oestrone and gonadotrophin levels were unaffected during treatment. Clinical and subjective improvement was apparent and acceptance of treatment was good.

1.9.1 Hormone influence on pelvic connective tissue in stress incontinence or prolapse

Chen et al examined fibroblasts from periurethral vaginal tissue of stress incontinent women in the secretory and proliferative phase of the cycle. It appeared that relaxin enhances elastolytic activity in stress urinary incontinence cells. It decreases the expression of alpha-1 antitrypsin and increases the total elastase activity in proliferative phase cells while increasing the total elastase activity in secretory phase cells. Fibroblast total elastase activity was inhibited by increasing concentrations of alpha-1 antitrypsin.[127] Relaxin is a peptide hormone and belongs to the insulin super family. It is produced by the corpus luteum of the ovary and during pregnancy by the placental tissue. The exact purpose of relaxin

in the female is not yet determined but it is mainly expressed in the luteal phase of the cycle diminishing if no pregnancy occurs and menstruation sets in. This could show a relation to hormonal influence on connective tissue in stress incontinent women.

Other studies fitting with these findings are again by Chen in 2003. This time the direct influence of oestrogen on protein expression by vaginal tissue fibroblasts of stress incontinent women was assessed. Findings were that TIMPs were decreased in tissue from incontinent women but no more dramatic oestrogen influence was noted. Fibroblasts from continent women showed a significant increase in TIMP expression with oestrogen. MMP2 expression was neither altered in incontinent women or controls.[128] Even though the influence of oestrogen on tissue from incontinent women could not be demonstrated it was effective in tissue from continent women. It could be possible that cellular manipulation prevented any response. Altogether, there is convincing evidence that a relation to hormonal influence on connective tissue in stress incontinent women exists.

1.10 Quality of Life

Pelvic organ prolapse has a significant impact on the quality of life. There is a large range of symptoms and women suffering from prolapse often seek help and advice initially because of these. It is rather difficult to establish how large the impact of these symptoms is on the normal quality of life. The King's Health questionnaire was developed to assess quality of life more accurately and give the possibility of a better subjective analysis.

It was developed by Digesu et al. and published in 2005.[129] The validity and the internal reliability showed good statistical outcomes. The test-retest reliability confirmed a highly significant correlation between the total scores for each domain. The P-QOL questionnaire for English-speaking patients was proven to be reliable and valid. Since then it was translated and tested in various non-English speaking countries and was found to be a consistent and valid instrument for assessing symptom severity, impact on quality of life in women with uterovaginal prolapse. It is easy to understand, may be easily administered and self-completed by the women.[130131132] In view of these attributes this questionnaire was chosen to measure the differences in quality of life prior and post three months after Vagifem application.

For other studies, Digesu et al examined women who presented with symptoms specific to pelvic organ prolapse. It was found that women who had significant symptoms also presented greater degrees of pelvic relaxation than women who presented without symptoms. Prolapse severity and quality of life scores were significantly different in those women symptomatic of prolapse. There was a stronger relationship between posterior prolapse and bowel symptoms than anterior prolapse and urinary symptoms. Sexual dysfunction was related to cervical descent.[133]

Fritel et al established that pelvic organ prolapse symptoms were associated with difficulty defecating, lower abdominal pain, and difficulty voiding. The frequency of POP symptoms was associated with a poorer quality-of-life score in the categories physical mobility, pain, emotional reaction, social isolation, energy, and sleep. In women above 50 years of age, POP symptoms are associated with impaired quality of life, and the number of vaginal deliveries is a risk factor for past or present symptomatic POP.[134]

Oestrogen would be given to improve prolapse symptoms and reduce the chances of the prolapse worsening, but ultimately long term assessment of pelvic floor protein alterations has to be measured in terms of improvements in quality of life.

1.11 Hypotheses and Objectives

The prevalence of stress incontinence (SI) in white women is much higher (61%) than in black African women (27%) and it has been suggested that the elasticity of vaginal skin is higher in black women and this could be the reason why black women are more protected against SI. Our research group and others have demonstrated changes in the collagen I:III ratios in the extracellular matrix of vaginal tissue in women with SI compared with controls. As the collagen III increases in respect to collagen I the tissue becomes more elastic. Our research group has also shown that collagen I and III ratios are different in continent black women compared to white and that the collagen III in respect to collagen I is higher.[135],[136] In addition it was found that the expression of collagen XVII, a collagen with adhesive properties was increased in black women compared with white. The above findings suggest better elasticity and adhesiveness in the tissue of black women. Thus, it is of interest, if an extracellular component with greater elastic characteristics such as elastin is also different in black women and changes in SI and pelvic organ prolapse (POP).

The mechanism of POP is poorly understood but it has been proposed that oestrogen might alleviate or improve some POP symptoms and or may influence surgical outcome. However, previous studies have not been conclusive in showing improvements in pelvic organ disorders, although oestrogen has been shown to increase pro-mmp2 and new collagen formation in stress incontinent women.[137] Although this latter study indicates a positive effect of oestrogen on vaginal tissue the use of other oestrogenic compounds has indicated a negative influence on POP; for use of raloxifene or levormeloxifene, selective oestrogen receptor modulators (SERM), acting partially as inducers or inhibitors of oestrogen receptor action, can result in symptomatic prolapse [138],[139] Therefore,

there is interest in future studies that shed light on how oestrogen action can play a part in alleviating pelvic organ prolapse conditions.

Hypotheses

It was hypothesised that:

a) there is a greater elastin content in black continent women's vaginal skin compared with white women
b) stress incontinent women have differing elastin content compared with continent controls

Through findings from studies related to above hypotheses it was further hypothesised that changes in the vaginal tissue of white women with POP could be initiated through the influence of topical, vaginal use of oestrogen:

These hypotheses are therefore :

1) Topical oestrogen will increase vaginal skin elasticity in white women with POP through
   a) increasing elastin mRNA expression and protein content
   b) lowering the collagen I:III ratio, as collagen III has more elastic properties

2) Topical oestrogen will
   a) increase MMP2 expression to remodel tissue and reduce stress,
   b) alter adhesive nature of the tissue for remodelling by altering collagen XVII expression,
   c) increase ER alpha expression to augment action

Objectives

1) The aim is to examine the elastin content of white and black women with or without stress incontinence. Periurethral vaginal tissue will be collected, processed and analysed by histochemistry.

2) To confirm the hypothesis of improving vaginal tissue characteristics with local oestrogen, the following study was designed:
Women with POP will be recruited, specimens will be taken and clinical symptoms recorded pre-treatment and after three months treatment with Vagifem®, 25µg 17-β-Oestradiol. Periurethral non stretched tissue will be obtained and examined.
The mRNA expression of MMP2, elastin, ER alpha and collagen XVII as well as the protein content of elastin and collagen I and III of vaginal skin will be assessed before and after topical oestrogen treatment.

Chapter 2 :

Study Design

2.1 Ethical approval and Data Protection

Ethical approval for the racial study was obtained by the Tygerberg Hospital Ethical Committee in Cape Town, South Africa, project number 2003/033. The ethical approval for the prolapse study was granted by the Southampton & South West Hampshire Local Research Ethics Committees, REC number 031/04/t

All patient's files were stored securely in the Urogynaecology Department, Princess Anne Hospital, University of Southampton, so that the patient's private data were protected at all time.

2.2 Racial Study

2.2.1 Study Population

Patients for the racial study were recruited form the gynaecology clinic at Tygerberg Hospital in Cape Town. Women awaiting surgery for stress incontinence and women awaiting other gynaecological procedures for benign conditions not suffering from stress incontinence were recruited given that they fulfilled inclusion and exclusion criteria.

2.2.2 Patient Recruitment, Inclusion and Exclusion Criteria

A detailed history was obtained of each patient recruited for the study including past obstetric history, gynaecological, medical and surgical history, as well as medication and allergies. Urinary symptoms were recorded.

Inclusion criteria

- age between 18-85 years
- white or black African ethnic group
- awaiting surgery for either stress incontinence
  or for other benign gynaecological conditions
- urodynamically proven stress urinary incontinence
- able to give written consent

Exclusion criteria

- no proven stress incontinence
- previous history of pelvic floor surgery
- history of irradiation
- medication including
    - Diuretics
    - ACE inhibitors
    - HIV or hepatitis B positive
- medical history including
    - chronic cough or constipation
    - connective tissue disorders
    - diabetes
    - haemorrhoids or varicose veins

- urinary tract infection
- CNS disorders or spinal cord injuries
- genitourinary anomalies or congenital disorders
- PID
- malignancy
- ligament injury

2.2.3 Patient Data

An abdominal and vaginal examination was performed to exclude pelvic abnormalities or prolapse and to assess vaginal tissue. The perineum was assessed and was classified as healthy, scarred or deficient. A cough test was performed to exclude stress incontinence symptoms. Routine blood test to exclude HIV or Hepatitis B infection was carried out. Women with urinary stress incontinence underwent urodynamic studies according to ICS guidelines. These included uroflowmetry to measure flow rate and reveal any underlying outflow obstruction and cystometry to assess intra vesical pressure and volume during bladder filling and voiding. If detrusor instability was demonstrated or urodynamic stress incontinence not demonstrated, women were taken out of the study.

2.2.4 Obtaining Tissue and Storage

Periurethral full thickness vaginal wall samples were obtained during surgery. A 10x10mm^2 biopsy was taken and divided into three sections. Two samples were fast frozen in liquid nitrogen and stored using the laboratory facilities at -80º C until their transport to the UK. The third sample was embedded in paraffin.

During transport to the UK it was ensured that the frozen samples were prevented from thawing with the use of dry ice. Regular controls were instigated and dry ice added if necessary.

All samples were encoded meticulously and in accordance to the patient questionnaire. The details of the patients matching the samples were not revealed to the investigator.

2.3 Prolapse Study

The patients for the prolapse study were recruited from the waiting list for an anterior and or posterior repair at the Princess Anne Hospital, University of Southampton. The women were informed in detail about the study and asked if they would give consent to participate. Women who fulfilled the inclusion and exclusion criteria and consented were recruited and filled in a data collection and symptom questionnaire particularly designed for the study and a Kings Health quality of life questionnaire.

2.3.1 Patient Recruitment

Women were only recruited for the prolapse study if they fulfilled inclusion and exclusion criteria.

Inclusion criteria

postmenopausal for at least two years

awaiting surgery for at least a stage 2 POP

Exclusion criteria

previous vaginal surgery at the planned operation site

history of malignancy

collagen diseases

stress incontinence

Women with these conditions were excluded as it is known that these predispositions could alter extracellular matrix component expression and turnover.

The study was designed initially as a randomised, double blind, placebo controlled study. The supplier of the Vagifem tablets initially produced the placebos accordingly but unfortunately the packs were inappropriate. As re-production was not an option for the company the placebo tablets had to be removed. The women receiving Vagifem in the present study are therefore acting as their own controls as pre-treatment samples and post-treatment samples were obtained and subsequently results compared. This design was considered appropriate in view of a previous study conducted by Jackson et al in 2002. In this study oestradiol valerate was compared to placebo to establish its influence

on vaginal tissue in postmenopausal stress incontinence women. Whilst synthesis of new collagen and production of pro-MMP2 was found after 6 months in the oestrogen treated women the placebo controls showed no significant effect[140].

In the present study, patients were asked to fill in a Kings Health Quality of Life (QoL) questionnaire and a Standardised Symptoms questionnaire. A detailed history including, age, weight, smoking and drinking habits, previous obstetric, surgical and medical history was obtained as well as a detailed gynaecological and urogynaecological history if present. Allergies and current medication were noted accordingly. The symptom sheet can be found in the appendix. A thorough abdominal examination was performed together with a vaginal and bimanual assessment, in addition to inspection with a Sims speculum. The assessment of the stage of prolapse was completed in accordance with the ICS standard (POP-Q score). A cough test was performed to rule out stress incontinence symptoms.

2.3.2 Obtaining and Storage of Tissue

Over the study period it was possible to recruit and see 16 women before and after treatment. The study group received a pelvic ultrasound scan to measure endometrial thickness and started treatment with topical vaginal oestrogens (25µg of 17-β-oestradiol pessaries once daily for 14 days and then 25µg twice weekly as recommended by the BNF).

Prior to oestrogen treatment a small full thickness biopsy (5mm x 5mm) from non-stretched vaginal skin of the vaginal wall involved in the prolapse was obtained under local anaesthetic from the patients in the study group. The tissue was

obtained using a scalpel to achieve even samples of good size. It is emphasised that the tissue was not stretched but was comprised in the prolapse as previous studies showed that protein activity and remodelling was significantly higher in non-stretched vaginal wall tissue compared to stretched vaginal wall tissue as Laborda et al demonstrated.[36]

The obtained tissue was washed quickly to remove blood components in Dulbecco's phosphate buffered saline (PBS) PBS 1X with Ca & Mg, Catalog No. 9236 with a pH of 7.4, containing the following components:

Dulbecco's PBS:	(mg/L)
- Potassium Chloride (KCl)	200
- Potassium Phosphate monobasic (KH_2PO_4)	200
- Sodium Chloride (NaCl)	8000
- Sodium Phosphate, dibasic (Na_2HPO_4)	1150
- Magnesium Chloride $6H_2O$ ($MgCl_2 6H_2O$)	100
- Calcium Chloride (CaCl)	100

The tissue was then divided into two pieces at approximately a 2:1 ratio. The larger piece was frozen rapidly in liquid nitrogen and transferred to the laboratory and stored at -80ºC until it was processed. The smaller piece was soaked in standardised 10% neutral buffered formalin solution for 24 to 48 hours, then transferred into 75% ethanol and transferred to the histopathology laboratory where the sample was embedded in paraffin blocks for cutting and staining. The same procedure was performed with the samples harvested during surgery. All samples were labelled clearly with the initials of the patient, date of birth, study number and collection date before storage.

Chapter 3 :

Histochemistry

3.1 Histochemistry and Immunocytochemistry

It both studies (racial comparison study and prolapse study) the staining of elastin protein was carried out by histochemistry.
However, for collagen I and III established immunocytochemistry techniques were used (prolapse study).

3.2 Fixation and Types of Fixatives

The purpose of fixation is to preserve tissues permanently in as life-like a state as possible. Fixation was carried out as soon as possible after removal of the tissues to prevent autolysis. Formaldehyde (formalin) was used which forms cross links in proteins, especially between lysine residues. These links do not change or deteriorate the structure of tissue proteins greatly, so that antigenicity is not lost or masked. Formaldehyde is ideal for immunostaining techniques. Penetration of the tissue is slow but distinct. The standard solution is 10% neutral buffered formalin. A buffer prevents acidity that would promote any autolysis. Other fixatives such as glutaraldehyde or mercurials do not penetrate tissue to the same degree as formalin or cause deformation of alpha-helix structures in proteins and so are not recommended for immunostaining. Both alcohols methanol and ethanol cause denaturation of proteins and so are not used for the fixation stage but washing in later processes.

Fixation should occur at a neutral pH (6-8) to prevent excessive acidity. Common buffers are phosphate, bicarbonate, cacodylate, and veronal. Formalin is buffered with phosphate at a pH of 7. There should be a 10:1 volume ratio of fixative to

tissue and increasing the temperature enhances the fixation process. Tissues for the prolapse study were left in phosphate buffered formalin at room temperature for 24 hrs.

Once fixation has taken place the tissue is processed into thin microscopic sections. Paraffin was used as a carrier. Paraffin is similar in density to tissue and can be cut into slices as thin as 3 micrometers. Tissue was dehydrated to be embedded in paraffin. Water was removed by dehydrating the tissue through graded alcohol in varying concentrations. (70% to 95% to 100%). Afterwards the tissue was “cleared” to remove the insoluble alcohol. Usually xylene, chloroform or clearene (aliphatic hydrocarbons) are used. In a final step the tissue was immersed in liquid paraffin. The "embedding" process should ensure that the tissues are aligned in the right orientation for cutting of the paraffin block and preparing the microscopy slides.

3.3 Cutting or Sectioning

Cutting was performed with a microtome steel blade set to a standard of 4µm advance. The sections were floated on warm water to eliminate possible creases. They were then picked up onto glass microscopic slides which were coated with aminopropyltriethoxysilane (APES). This adhesive provides stability and avoids movement of the tissue section during further processing. The slides were stored over night at 37°C and then left at room temperature. All slides were labelled accordingly similar to the sample cryo tubes.

3.4 Immunocytochemistry Methods for Collagen I and Collagen III

3.4.1 Immunoperoxidase Staining

An antibody reaction was used to detect specific protein (Collagen I and collagen III). These antibodies can be monoclonal or polyclonal. (Monoclonal antibodies derive from cell cultures and often only bind to one protein epitope. Polyclonal antibodies are obtained from animals and so form a heterogeneous blend of antibodies able to detect a variety of protein epitopes.) Direct or indirect probing can be performed using one or two types of antibodies. The latter is called the "sandwich" technique and was used in this study. The sandwich technique is used to ensure an enhanced reaction compared to a direct antibody staining. In the direct method a customised antibody would be required for any variety of antigens of interest.

In the Indirect immunoperoxidase staining technique, the specific (primary) antibody was bound to the tissue sample and then coupled with a tagged secondary antibody carrying small molecules that can be recognised by a peroxidase-conjugated binding molecule with high affinity. The most common example for this is a biotin linked antibody that binds to an enzyme-bound streptavidin. This method can be used to amplify the signal.

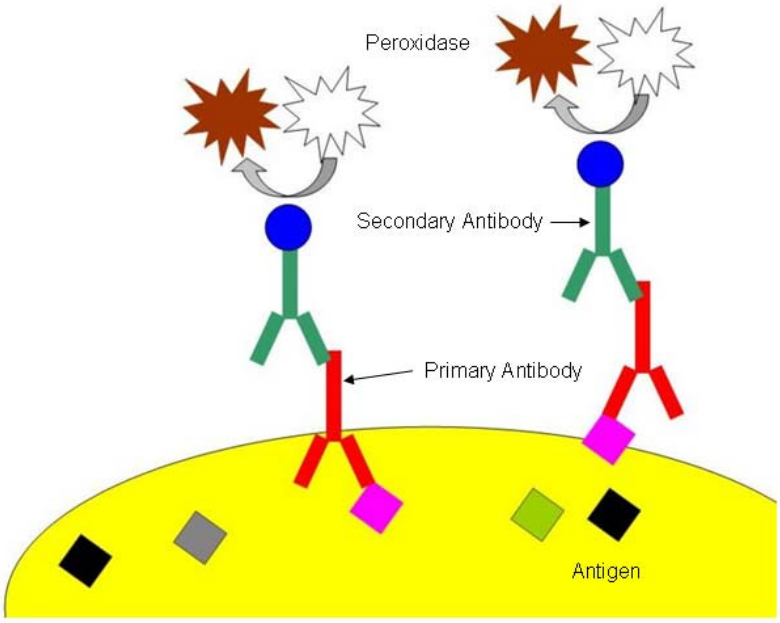

Figure 3.1 Scheme of peroxidase staining, using primary and secondary antibodies

Detection is typically performed using the peroxidase linked antibodies. Biotinylated secondary antibodies were coupled with peroxidase which reacts with 3,3'-diaminobenzidine (DAB) and produces brown staining. The reaction can be enhanced using nickel, producing a deep purple/grey staining.[141] The amount of colour produced is equivalent to the quantity of enzyme and therefore equivalent to the amount of tissue antigen that is to be assessed.

To ensure that only the specific reactions are detected all other innate enzymes which could produce similar colour changes needed to be inactivated. Otherwise falsification of results would occur. For this 0.5% hydrogen peroxidase in methanol was used for ten minutes to ensure inhibition. The tissue was then washed three times with Tris buffered saline (TBS) for two minutes.

3.4.2 Antigen Retrieval

Through formalin fixation and paraffin embedding, antigen sites can be hidden and need to be unmasked. For this Heat Induced Epitope Retrieval (HIER) was used to break the cross links formed by formalin fixation. This technique involved heating the paraffin-embedded tissue for varying lengths of time in an aqueous solution. (Retrieval solution). Hidden antigenic sites are recovered and the staining reaction is enhanced.

3.4.3 Antibodies

Primary antibodies used were -

1. for collagen I, a mouse monoclonal from Abcam (Cambridge, UK)
2. for collagen III, a rabbit polyclonal from Cedarlane Laboratories, (Canada)

The tissue was incubated with the primary antibodies overnight at 4°C and washed three times for 5 minutes in TBS. Biotinylated secondary antibodies were used to build a primary-secondary- antibody complex and the slide was incubated for 30 minutes.

Secondary antibody used

3. for collagen I: anti-mouse IgG
4. for collagen III: anti rabbit IgG

Slides were then washed again three times using TBS.

3.4.4 High Affinity Reaction

Streptavidin- Biotin-peroxidase complexes (ABC, by Dakocytomation, Ely,UK) were applied for 30 minutes for a high affinity interaction. Streptavidin is a tetrameric 60kD Avidin analogue isolated from Streptomyces avidinii. It has a high affinity to biotin (106 times higher compared to normal antibody-antigen binding) and is able to bind four biotin molecules simultaneously. The biotin is conjugated to the peroxidase enzyme. Thus it amplifies the antibody-antigen complex formation.

The chromogen used in this study was DAB (as described above). It is insoluble in alcohol and so achieves permanent, brown staining. It was applied for 5 minutes and washed with TBS and water. Counter staining was undertaken with Mayers haematoxylin blue dye. The slides were dehydrated with graded concentrations of alcohol, washed with xylene (phenol solvent) and mounted in Di-n-butyl Phthalate (DPX). Subsequently they were left at room temperature for analysis.

3.5 Elastin

Imaging of elastin fibres in vaginal tissue does not necessarily require an antibody binding process as does collagen imaging. Staining with elastic van Gieson (EVG) method was performed for samples of the stress incontinence and the prolapse study.

Samples were embedded in paraffin and 4µm sections cut and floated on warm water. They were then floated onto glass slides, coated with aminopropyltriethoxysilane (APES) providing an adhesive for the tissue to obtain the histological correctness and dried overnight at 37°C.

The elastic fibres were then stained by the Elastic van Gieson method. Sections were dewaxed in Clearene, and rehydrated through decreasing concentrations of alcohol to water and then treated in 0.5% Potassium permanganate for 5 minutes. They were then rinsed in distilled water and bleached in 1% Oxalic acid for 3 minutes, rinsed in distilled water and then in 95% alcohol. They were then stained with Millers Elastin Stain for 3 hours. (A combination of Victoria Blue, New Fuchsin, Crystal Violet, Resorcinol, Dextrin and Ferric chloride. Excess stain was removed with 95% alcohol, and the sections washed in running water for 2 minutes. The nuclei were then stained with Weigert's iron haematoxylin (1% alcoholic haematoxylin in acidified ferric chloride) for 5 minutes. They were then washed in running tap water to "blue" the nuclei, and then counterstained with Van Gieson stain for 5 minutes (10 ml 1% aqueous acid fuchsin + 100ml saturated aqueous picric acid. The sections were then dehydrated through increasing grades of alchohol, "cleared" in clearene and mounted in a synthetic mounting medium (eg DPX, pertex, etc.)

Elastic fibres are presented blue/black, cell nuclei black, muscle tissue yellow, and connective tissue red.

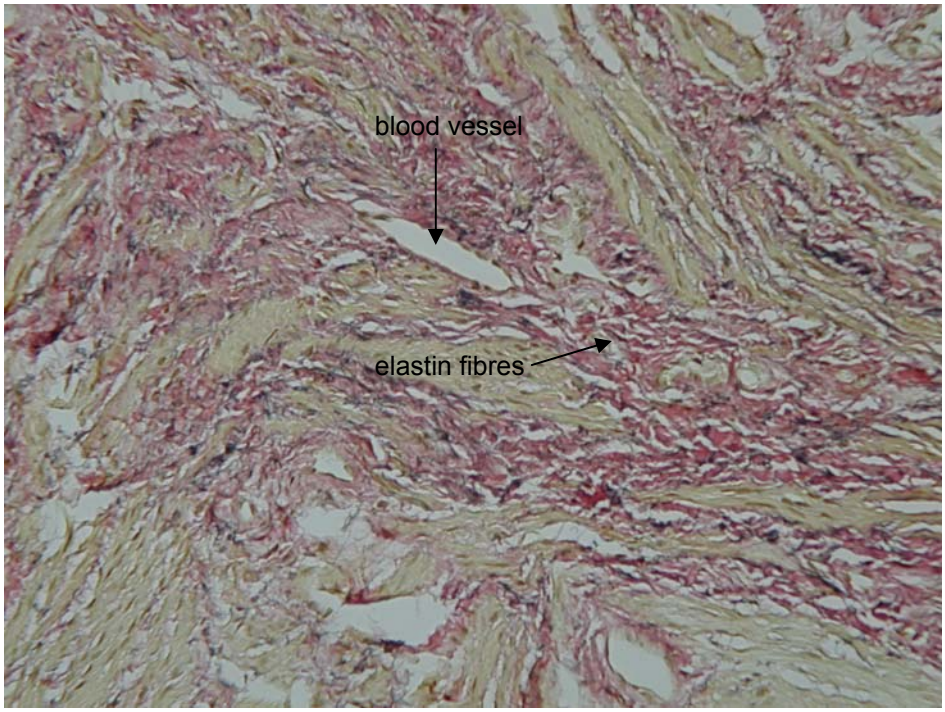

Figure 3.2 Elastin fibres in Elastic van Gieson staining. The fibres appear black meanwhile collagen fibres are red stained. (Amplification x40).

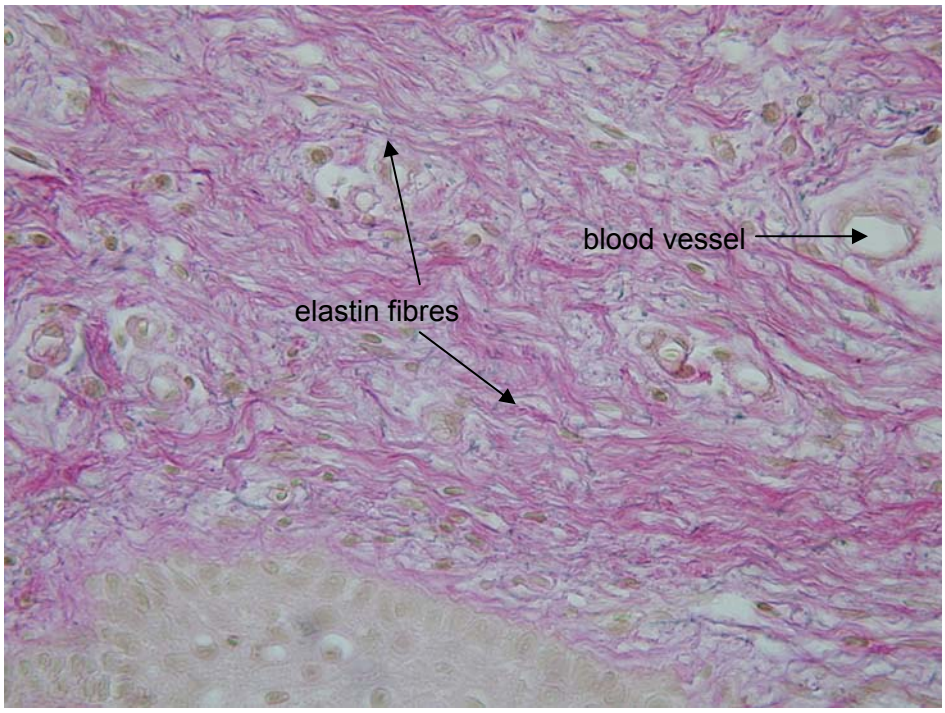

Figure 3.3 Elastin Fibres in Elastic van Gieson Staining in another sample. Less fibres are present (Amplification x40).

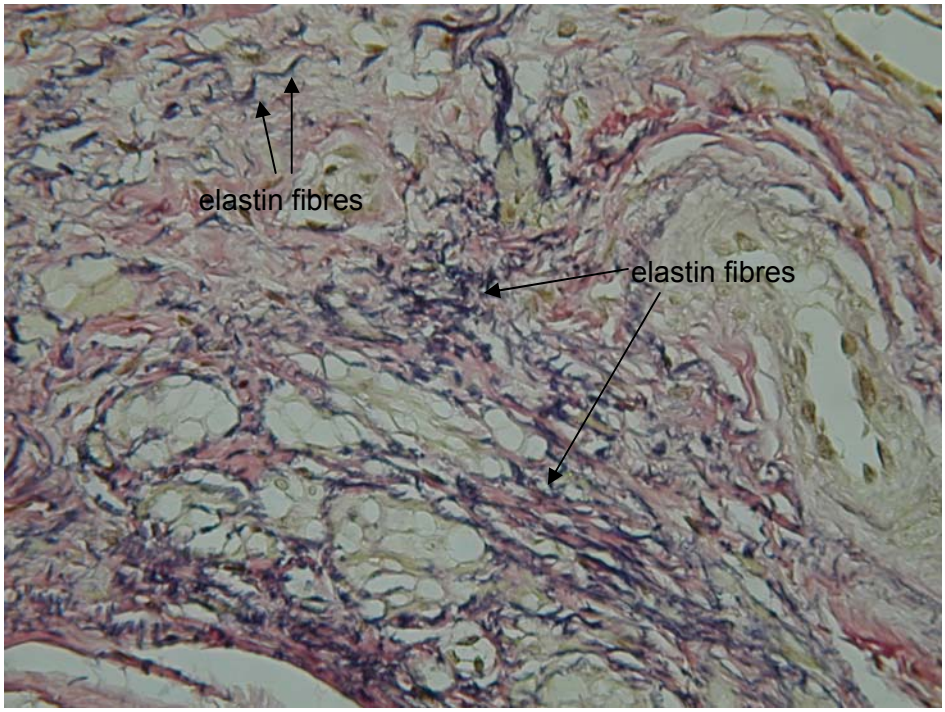

Figure 3.4 Elastic van Gieson Staining in a sample with a high percentage of elastin fibres. (black), (Amplification x40)

An image analysis system by Zeiss was used to analyse the microscopic slides for collagen I, III and elastin. (Zeiss KS400 software, Image Associates, Bicester, UK). The system includes a microscope (Zeiss Axioskope 2 MOT) with an attached camera (Zeiss Axiocam) and connection to a computer with the required program to analyse the measured data (KS400.30 from Image Associates). Each slide was assessed to ensure morphological correctness and the different layers of vaginal skin were identified prior to measurement. Three to five sections of each slide were randomly chosen and areas of artefact or vessels were omitted using the computer system before the percentage content of Collagen I or Collagen III was assessed based on the red, green, blue colour composition. The average of each of the three sections was calculated and taken as overall percentage content for that particular slide.

Collagen ratio was then calculated by dividing the percentage of collagen I content by the percentage collagen III content.

$$\frac{\text{Collagen I (\%)}}{\text{Collagen III (\%)}} = \text{Ratio collagen I/III}$$

The percentage elastin content (grey/black staining) was measured alike using the above mentioned computer-assisted image analysis based on its red-green-blue colour balance. Reproducibility was assured by repeatedly measuring the same areas in a number of slides and calculating the coefficient variation of the results which varied between 7 and 10%.

$$CV\ \% = \frac{\text{standard deviation of the mean}}{\text{mean}} \times 100$$

Chapter 4 :

Molecular Biology Methodology

4.1 Techniques for Homogenizing Tissue Samples for Real Time PCR

The tissues of the racial and the prolapse study were processed in the same way. The techniques therefore apply for both studies.

4.1.1 Cutting of the Samples

The samples were removed from the -80º C freezer and stored immediately on dry ice. The samples were cut in a sterile plastic petri dish to prevent contamination and on dry ice to prevent thawing. All other instruments used (forceps, blade, spatula, sample container) were sterile and cooled on dry ice. Afterwards, samples were weighed using a calibrated, sensitive sheltered balance and the sample weights were approximately 50mg.

The samples were kept individually in labelled cryo tubes on dry ice and were then homogenized using 500µl of TRIzol® as recommended by "Invitrogen life technologies" instructions for the use of Trizol. TRIzol® is a mono-phasic solution of phenol and guanidine isothiocyanate and is used for the isolation of total RNA from cells and tissues. It maintains the integrity of the RNA while disrupting cells and dissolving cell components. It has the advantage to have a broad spectrum of efficacy and can be used for tissue quantities from a few mg up to 1g. It can be used for isolation of a variety of RNA species of large or small molecular size. The isolated RNA has an A_{260}/A_{280} ratio of ≥1.8 when diluted into TE buffer.[142]

4.1.2 Homogenization Techniques

After grinding the tissue to powder in a pulveriser cooled by liquid nitrogen, the tissue powder was retrieved with a sterile spatula and transferred instantly into 500µl TRIzol® and was then homogenized. Homogenization was performed with a Labour technik T8.01 Ultra-Turrax homogenizer by IKA Werke GmbH & co.KG, Staufen, Germany, using 15 sec bursts on ice so that the tissue did not overheat.

All metal parts were thoroughly cleaned between samples with water and 100% Ethanol to stop cross over contamination and cooled again to prevent thawing. Comparative trials using only the Ultra-Turrax homogenizer, and both the grinder and homogenizer in combination showed that the RNA concentration was considerably higher in the sample that was ground and homogenized.

Example –

Sample	A260	A280	A260/280	RNA Concentration
1 homogenized AND ground	0.067	0.048	1.895	0.67µg/µl
2 homogenized only	0.034	0.026	1.813	0.34 µg/µl

Table 4.1 Calculation for RNA quantification and the method of spectro-photometry follows later.

4.1.3 RNA Extraction and Quantification of RNA

Once the tissue was ground and homogenised it was left in the TRIzol® reagent to incubate for 30 min at room temperature to permit the complete dissociation of nucleoprotein complexes.

The following steps were then used for RNA extraction using TRIzol® reagent:

1) 100µl of Chloroform were added (200µl per 1ml of TRIzol®)
2) Mixture shaken thoroughly for 15 sec
3) Incubated at room temperature for 5 min
4) Centrifuged at 12.000rpm for 10 min

Following centrifugation the mixture separated into a lower, red phenol-chloroform phase, a white interphase and a clear upper aqueous phase.

5) the top layer was transferred (supernatant aqueous phase) to a clean, labelled tube, ensuring that only the clear top layer was used and the other, lower layers remained undisturbed as this would have contaminated the RNA-rich supernatant.
6) 20µl of Glycogen (concentration 2mg/ml) was added to aid precipitation
7) 250µl of isopropylalcohol was then added (500µl per 1 ml of TRIzol®)
8) It was left on ice for 10 min
9) Precipitate was centrifuged down for 10 min at 12.000rpm
10) Supernatant was removed carefully to leave small pellet of glycogen and RNA
11) Pellet was washed with 500µl of 75% ethanol

12) After mixing the precipitate was centrifuged down at 12.000rpm for 10 min

13) Ethanol was then removed and pellet allowed to dry

14) The RNA was dissolved in 20µl of ultra pure water

15) The RNA concentration was measured in a spectrophotometer and quality also assessed by Agarose gel electrophoresis to assess 18s and 28s ribosomal bands.

4.1.4 Quantification of RNA

A Jenway 6505 UV/vis (UK) spectrophotometer was used

Figure 4.1 Jenway 6505 UV/visuel (UK) Spectrophotometer.

It is a single beam spectrophotometer having a 1.8nm bandpass and offering spectrum scanning, quantitation, kinetics and multi-wavelength measurements. It operates between 190-1100nm.

RNA absorbs light mainly at 260nm whereas protein absorbs light at 280nm, but RNA absorption overlaps into the 280nm band. These characteristics are used to quantify RNA content in a sample in relation to protein content (A260/A280). If there is over 95% RNA content with little protein contamination then the ratio should be between 1.5 and 2. Lower ratios would mean that the sample is contaminated and not valid for further usage.

To test a sample 2µl were taken initially and used for spectrophotometry. If readings were too low then 10µl of extracted RNA were used and diluted with 500µl of purified water in the instrument's cuvettes. After mixing, the cuvette was placed in the spectrophotometer and measured using the multiwavelength setting.

RNA concentration was calculated using the principle that if A260 is equal to 1, through a 1cm path (thickness of the cuvette), then the concentration is equivalent to 40µg RNA per ml.

Cuvette	volume	500	ul			
RNA soln.	volume	2	ul			
Abs	A260	0.104	so RNA	4.16	ug/ml	in cuvette
cuvette	total RNA	2.08	ug			
therefore	RNA conc	1.04	ug/ul	***		
vol. orig	RNA soln.	20	ul			
total RNA	orig. soln.	20.8	ug			

Table 4.2 Calculation scheme for RNA concentration

The example above refers to a sample of 2µl RNA in 500µl water with A260 as 0.104nm giving a concentration of 4.16µg/ml

(0.104 x 40µg/ml = 4.16µg/ml, i.e. 2.06µg in 2µl diluted in 500µl water in cuvette).

4.1.5 Dilution of RNA

The samples used for a PCR assay contained similar concentrations before they were processed in further steps to ensure valid and comparable data. The assays were optimised so that expression of a particular gene fell within the range of the assay. The RNA was diluted to an approximate concentration of 0.5 ug/µl.

4.1.6 Agarose Gel

The purity of RNA is tested using Agarose gels before cDNA is made. The RNA for 18s and 28s is looked for to assess the quality.

Details for the gel electrophoresis:

500mg agarose was dissolved in 50 ml of 1 x TAE buffer by heating in a microwave oven to produce a 1% solution. While still in solution 1.5µl of ethidium bromide solution (10mg/ml) was mixed into the agarose solution, which was then poured into a gel holder and a comb placed in the gel before allowing to set at room temperature

RNA samples were made up in the following mixture before loading into the gel:

2µl of RNA solution

2µl deionised formamide

1µl Northern loading buffer

5µl RNAse free water

Samples were then run in the gel at a voltage of 10volts/cm across the gel for 30 min. The gel was then visualized under a UV light and a photograph taken of the gel.

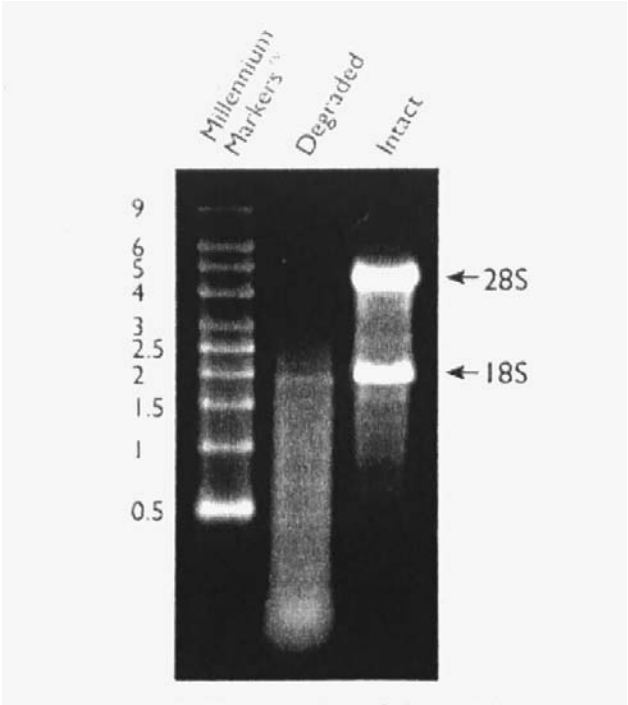

Figure 4.2: Intact versus degradred RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millenium Markers on a 1,5% denaturating Agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNa sample. The degraded RNA appears as a lower molecular weight smear

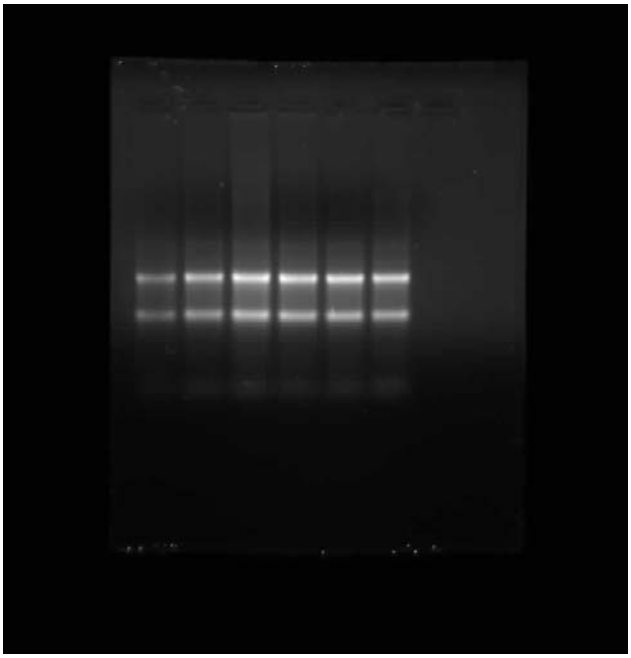

Figure 4.3: Typical picture of agarose gel with intact and clear RNA samples

4.1.7 Reverse transcription

To quantify the expression of a gene by PCR the RNA had to be reverse transcribed into complementary DNA (cDNA) and then the region of interest was amplified using PCR. Reverse transcription followed by PCR was used in preference to Northern blot analysis or RNAse protection assay, as RT-PCR is able to quantify much smaller amounts of RNA.

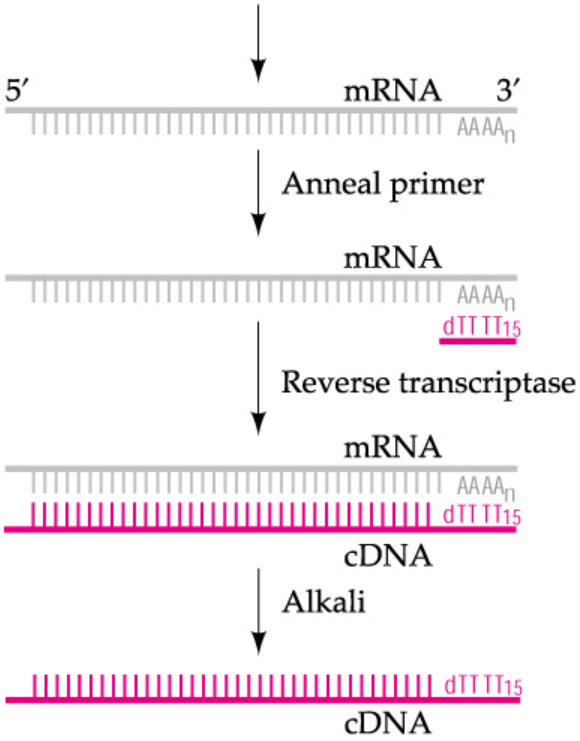

Figure 4.4 Reverse Transcription Process Diagram

Reverse transcription was performed using M-MLV transcriptase (200U/µl) from Promega, Southampton (Code M1701). Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) is an RNA-dependent DNA polymerase that can be used in cDNA synthesis with long messenger RNA templates (>5kb). It is preferred because the RNase H activity of M-MLV RT is weaker than the commonly used Avian Myeloblastosis Virus (AMV) reverse transcriptase.[143]

M-MLV Reverse Transcriptase is supplied in 20mM Tris-HCl (pH 7.5), 200mM NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol. M-MLV RT 5X Reaction Buffer is diluted 1:5 and contains the following:

250mM Tris-HCl (pH 8.3 at 25°C)

375mM KCl

15mM MgCl2

50mM DTT

M-MLV Reverse Transcriptase is less active than AMV Reverse Transcriptase, and therefore, more units (1 unit is defined as the amount of enzyme required to catalyze the transfer of 1nmol of deoxynucleotide into acid-precipitable material in 10 minutes at 37°C) of the M-MLV enzyme are required to generate the same amount of cDNA as in the AMV reaction. Starting with 1µg of mRNA in a first-strand cDNA synthesis, 200 units of the M-MLV enzyme were used.

The following procedure used 2µl (1µg) of RNA. Initially random primers (500ng/µl) were diluted with ultrapure water in a ratio of 0.8µl:12.2µl and 2µl of RNA are added giving a total volume of 15µl per sample of RNA. A sterile RNase-free microcentrifuge tube was used for this. The mixture was heated at 70ºC for 5 minutes to open up secondary structure within the template and then cooled on ice for the random primers to anneal to the template. This was mixture 1. In the meantime further RT reagents were mixed (amounts below were for 1 sample and needed to be multiplied according to the sample number=n used.)

1) RT buffer		5µl
2) dNTPs (PCR mix 10mM)		1.25µl
containing		
dATP, 10mM		
dCTP, 10mM		
dGTP, 10mM		
dTTP, 10mM		
3) recombinant RNasin®		
Ribonuclease Inhibitor (40U/µl)	-	0.625µl
4) M-MLV transcriptase (200U/µl)	-	1.0µl
5) ultra pure water	-	2.125µl
Making a total volume of:		10µl

This second mixture was mixed gently and added to mixture 1 after the cooling stage, then mixed again and centrifuged.

A preset program for the reverse transcription reaction was run on a Hybaid thermal reactor as follows:

1 hour at 37°C

10 minutes at 42°C

10 minutes at 75°C (to denature enzyme and stop reaction)[144],[145]

4.1.8 Polymerase Chain Reaction (PCR)

The mRNA expression of oestrogen receptor α, MMP2, elastin and collagen XVII in the prolapse study were measured using real time PCR. The technique was similar for each of the genes but primer optimisation was performed prior to testing. The different measurements are described separately to signify the regions of interest and the primers and probes used.

The basic principle of the polymerase chain reaction for mRNA expression is to amplify cDNA representing the mRNA of interest and not genomic DNA that can contain both exons (parts of the gene coding for the mRNA and thus protein) and introns (areas between exons with no coding message). As introns are spliced out in the production of mRNA, primers can be designed to go over exon-exon junctions so that any contaminating DNA will not be amplified. The PCR amplifies a specific sequence representing the mRNA of interest. mRNA sequences were obtained from the National Center for Biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov/

The primers and probes were designed using the Applied Biosystems software Primer Express. As DNA is made of two complementary strands, a forward and reverse primer are necessary to read in either direction: from 5' end to 3' end of each DNA strand. For this the DNA strands is separated, a process called denaturation requiring high temperatures at around 95°C to take place. The temperature is lowered for the primer to bind and complement one of the DNA strands, a process referred to as annealing. Taq polymerase, an enzyme that catalyzes the polymerization of deoxyribonucleotides into a DNA strand, builds the complementary DNA strand using deoxynucleoside triphosphates (dNTPs) from the annealed primer (at the same temperature or raised to 72ºC). This step is called extension step.

The PCR usually consists of a series of 20 to 40 repeated temperature changes (or cycles) to increase the amount of DNA produced exponentially. The temperatures used and the length of time they are operated in each cycle depend on the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature of the primers.[146] Temperatures recommended for the different steps are:

Denaturation step

The DNA is heated 94-98°C for 20-30 seconds. This starts the melting of the DNA template and primers by disrupting the hydrogen bonds.

Annealing step

The reaction temperature is 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. The polymerase binds to the primer-template complex and starts the DNA synthesis.

Elongation step

The polymerase used for this step determines the temperature. The new complementary DNA to the template is built by adding dNTPs. The velocity of the elongation depends again on the polymerase used and the length of the DNA sequence being amplified. For real-time PCR, polymerases have been produced that can work at 60ºC and therefore annealing and extension are carried out at this temperature. The PCR reaction is then made much faster. The primers and probes are designed appropriately to work at this temperatue of 60ºC.

Cooling step (final hold)

The cycles can be stopped and the DNA preserved at 4-15°C for short-term storage of the reaction.

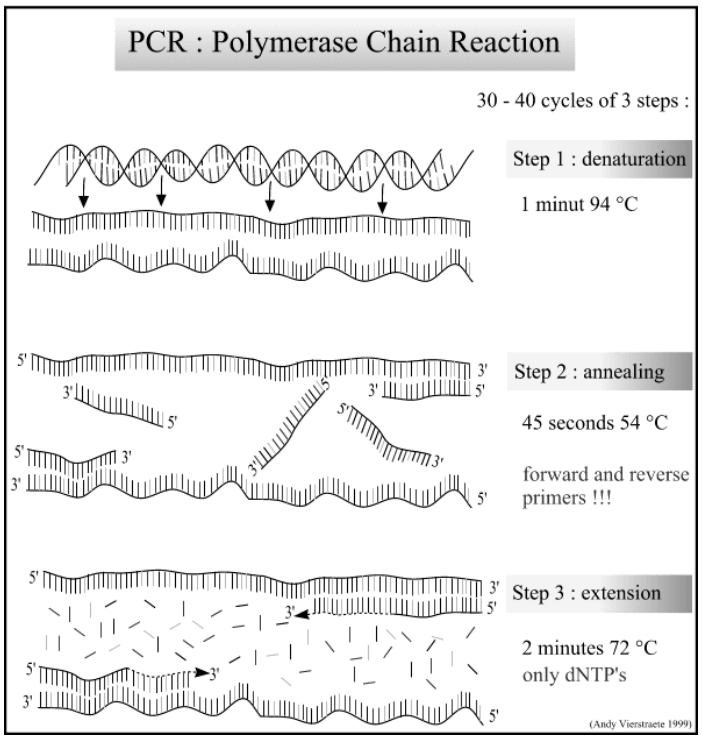

Figure 4.5 Schematic Drawing of a typical PCR Cycle.

Step 1: Denaturing at 94-96°C S

Step 2: Annealing at ~65°C

Step 3: Elongation at 72°C for several Cycles

For the real-time cycle – After denaturation, both annealing and extension occurs at 60ºC and therefore there are only 2 steps. In all of the following tests real time PCR was used. Real time PCR is also known as “quantitative real time polymerase chain reaction (Q-PCR)” or “kinetic polymerase chain reaction”. It provides the possibility of detection and quantification of a specific sequence of DNA in a sample.

The main difference to endpoint PCR is that the amplified DNA can be quantified in real time in the exponential phase of DNA amplification. Fluorescent DNA oligonucleotide probes with a quencher at one end are used that hybridise with the complementary DNA in the areas of interest between the primer areas. As the polymerase copies a DNA strand it digests the probe and releases the quencher from the fluorescent dye, whose fluorescent signal is then recorded. This fluorescent signal is proportional to the amount of DNA being produced. The probe contains a high energy dye, called a “Reporter” at the 5’ end, (often 6-carbofluorescein is used) and a low energy “Quencher” at the 3’ end (6-carboxy-tetramethyl-rhodamine). The quencher prevents fluorescence from being produced as long as the probe is intact.

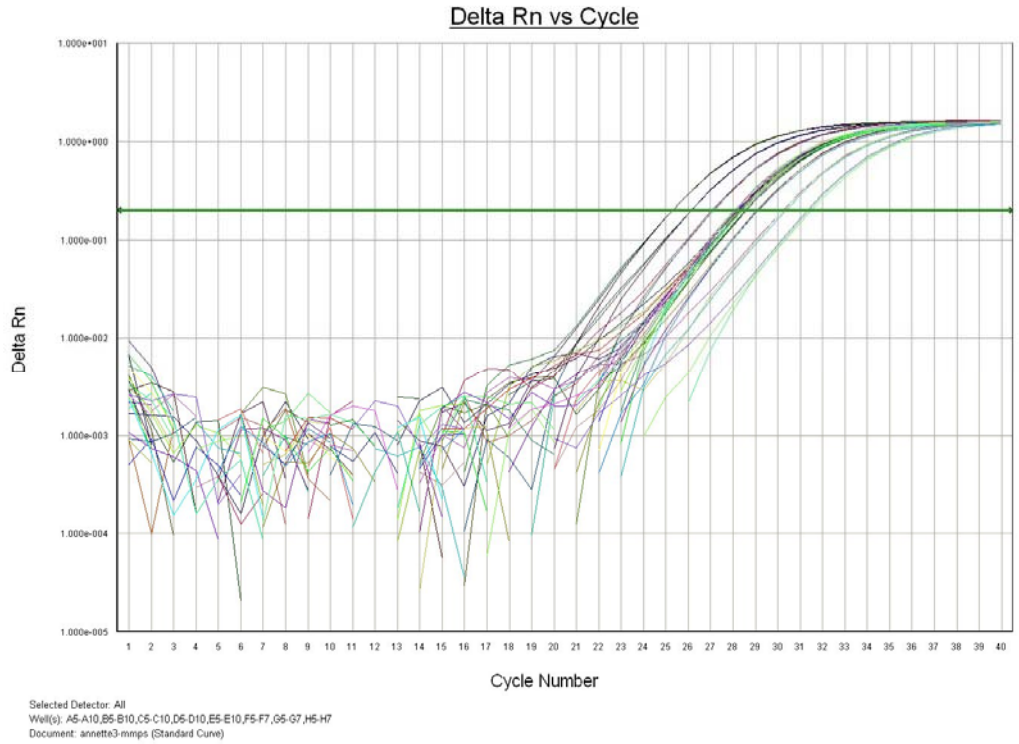

Figure 4.6 Cycle Threshold (Ct)(green line) for cycle measurement for fluorescence for standard and controls of assay for MMP2 in prolapse study

During the reaction the change in fluorescence is plotted against the cycle number on a semi-logarithmic scale (Figure 4.6). A threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold (Ct). A standard curve with known amounts of starting DNA (or mRNA) or dilutions of mRNA (arbitrary amount) can then be constructed from Ct values and known amounts of DNA. On a semi logarithmic graph this gives a straight line (see Figure 4.7)

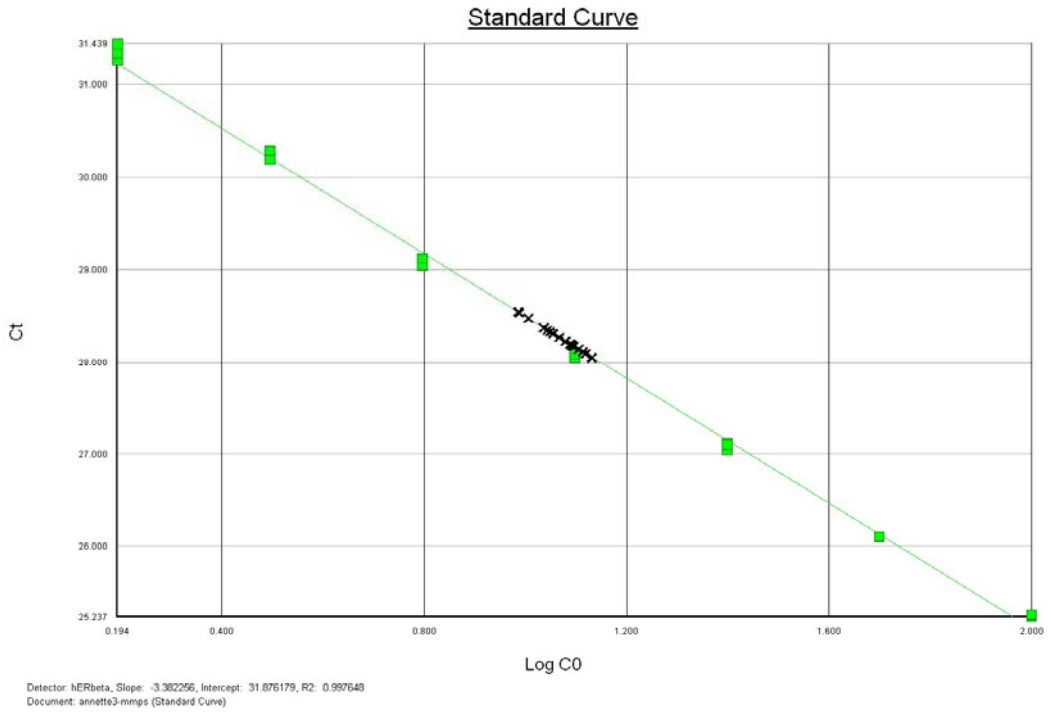

Figure 4.7 Logarithmic Graph of Standard Curve for MMP2 RNA

Unknown amounts of mRNA or DNA are then determined by reading the results off of the standard curve. The real-time systems used were the Applied Biosystems 7700 and later the 7500 with the ABI Prism Sequence Detection System software.

The results were calculated in relation to the expression of an internal control or housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH or G3PDH). GAPDH is an enzyme that catalyses the sixth step of glycolysis and thus serves to breakdown glucose. This gene belongs to the group of standard housekeeping genes. The RNA of housekeeping genes are produced regularly to maintain cell function continually and thus are generally unaltered dramatically when external stimuli change. Therefore when expression of genes of interest change, it is more accurate to measure them relative to this internal control that

has been taken through all the molecular biology techniques. Dividing the calculated mRNA amount for gene of interest by the amount of RNA from the house keeping gene measured in the same sample normalises the results for possible variation in the amount and quality of RNA between different samples. This normalisation ensures that the expression of the gene of interest can be accurately compared between different samples given that the expression of the housekeeping gene used in the normalisation is similar for all the samples. It is essential to use a housekeeping gene that is fulfilling this criterion.

4.1.9 Elastin Genome, Primer and Probe

Elastin mRNA expression was measured for both studies, Racial differences in stress incontinence and oestrogen influence on prolapsed vaginal tissue. The elastin gene can be found in the proximal region of the long arm of chromosome 7.[147]

The primers and probe were designed using primer express with the mRNA sequence for elastin, Accesion number NM_000501, and designed to pick up a common part of all alternatively spliced elastin forms. These were designated h-eln-f, h-eln-r and h-eln-p

The gene sequences were written from 5’ to 3’

Forward primer h-eln-f:

CCA-GGT-GTA-GGT-CGA-GCT-TTT-G

Reverse primer h-eln-r:

CCC-AGT-GGG-ACT-CCA-GGT-T

Probe h-eln-p:

CAA-AGG-GTC-CAA-CTC-CTG-GGA-TTC-CA

and manufactured by EUROGENTEC, Belgium: www.eurogentec.com

Homo Sapiens Elastin Gene Nucleotide Sequence

```
ORIGIN

1    gcgggctggg gcatttctcc ccgagatggc gggtctgacg gcggcggccc cgcggcccgg
61   agtcctcctg ctcctgctgt ccatcctcca cccctctcgg cctggagggg tccctggggc
121  cattcctggt ggagttcctg gaggagtctt ttatccagcg ctggggcctg gaggcaaacc
181  tcttaagcca gttcccggag ggcttgcggg tgctggcctt ggggcagggc tcggcgcctt
241  ccccgcagtt acctttccgg gggctctggt gcctggtgga gtggctgacg ctgctgcagc
301  ctataaagct gctaaggctg gcgctgggct tggtggtgtc ccaggagttg gtggcttagg
361  agtgtctgca ggtgcggtgg ttcctcagcc tggagccgga gtgaagcctg ggaaagtgcc
421  gggtgtgggg ctgccaggtg tatacccagg tggcgtgctc ccaggagctc ggttccccgg
481  tgtgggggtg ctccctggag ttcccactgg agcaggagtt aagcccaagg ctccaggtgt
541  aggtggagct tttgctggaa tcccaggagt tggacccttt ggggggaccgc aacctggagt
601  cccactgggg tatcccatca aggcccccaa gctgcctggt ggctatggac tgccctacac
661  cacagggaaa ctgccctatg gctatgggcc cggaggagtg gctggtgcag cgggcaaggc
721  tggttaccca acagggacag gggttggccc ccaggcagca gcagcagcgg cagctaaagc
781  agcagcaaag ttcggtgctg gagcagccgg agtcctccct ggtgttggag gggctggtgt
841  tcctggcgtg cctggggcaa ttcctggaat tggaggcatc gcaggcgttg ggactccagc
901  tgcagctgca gctgcagcag cagccgctaa ggcagccaag tatggagctg ctgcaggctt
961  agtgcctggt gggccaggct ttggcccggg agtagttggt gtcccaggag ctggcgttcc
1021 aggtgttggt gtcccaggag ctgggattcc agttgtccca ggtgctggga tcccaggtgc
1081 tgcggttcca ggggttgtgt caccagaagc agctgctaag gcagctgcaa aggcagccaa
1141 atacggggcc aggcccggag tcggagttgg aggcattcct acttacgggg ttggagctgg
1201 gggctttccc ggctttggtg tcggagtcgg aggtatccct ggagtcgcag gtgtccctgg
1261 tgtcggaggt gttcccggag tcggaggtgt cccgggagtt ggcatttccc ccgaagctca
1321 ggcagcagct gccgccaagg ctgccaagta cggagtgggg accccagcag ctgcagctgc
1381 taaagcagcc gccaaagccg cccagtttgg gttagttcct ggtgtcggcg tggctcctgg
1441 agttggcgtg gctcctggtg tcggtgtggc tcctggagtt ggcttggctc ctggagttgg
1501 cgtggctcct ggagttggtg tggctcctgg cgttggcgtg gctcccggca ttggccctgg
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1681 tggagttggt gctggtgttc ctggacttgg agttggtgct ggtgttcctg gcttcggggc
1741 agtacctgga gccctggctg ccgctaaagc agccaaatat ggagcagcag tgcctggggt
1801 ccttggaggg ctcggggctc tcggtggagt aggcatccca ggcggtgtgg tgggagccgg
1861 acccgccgcc gccgctgccg cagccaaagc tgctgccaaa gccgcccagt ttggcctagt
1921 gggagccgct gggctcggag gactcggagt cggagggctt ggagttccag gtgttggggg
1981 ccttggaggt atacctccag ctgcagccgc taaagcagct aaatacggtg ctgctggcct
2041 tggaggtgtc ctagggggtg ccgggcagtt cccacttgga ggagtggcag caagacctgg
2101 cttcggattg tctcccattt tcccaggtgg ggcctgcctg gggaaagctt gtggccggaa
2161 gagaaaatga gcttcctagg acccctgact cacgacctca tcaacgttgg tgctactgct
2221 tggtggagaa tgtaaaccct ttgtaacccc atcccatgcc cctccgactc cccaccccag
2281 gagggaacgg gcaggccggg cggccttgca gatccacagg gcaaggaaac aagaggggag
2341 cggccaagtg ccccgaccag gaggcccctt acttcagagg caagggccat gtggtcctgg
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2641 agcacctgaa gcctcaaagc tggattcgct ctagcatccc tcctctcctg ggtccacttg
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2941 atcctacact cccccagggc gtgcggggct gtgcagactg gggtgccagg catctcctcc
3001 ccacccgggg tgtccccaca tgcagtactg tatacccccc atccctccct cggtccactg
3061 aacttcagag cagttcccat tcctgccccg cccatctttt tgtgtctcgc tgtgatagat
3121 caataaatat tttatttttt gtcctgg
```

Figure 4.8 Homo sapiens mRNA for Elastin precursor variant protein, Accession number: AB208942 Totoki,Y., Toyoda,A., Takeda,T., Sakaki,Y., Tanaka,A., Yokoyama,S., Ohara,O., Nagase,T. and Kikuno,R.F. TITLE Homo sapiens protein coding cDNA, JOURNAL Published Only

(Yellow - forward primer; Green- reverse primer; Red - probe)

4.1.10 PCR for Elastin mRNA Expression

A standard curve was set up initially to ensure pipetting and accuracy of the measurement. The sample used (V87) showed RNA concentration of 0.98µg/µl (A260: 0.098)

Standard curve dilution was performed as per protocol:

Step 1) RNA made up to 20ul at a concentration of 0.5µg/µl (S7 standard)

Step 2) double dilution with 10µl water per sample until S1

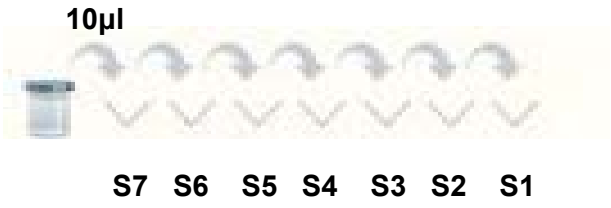

Figure 4.9 Dilution for producing a Standard Curve

Step 3) 3µl taken from S7 diluted with 21µl water for controls (C1 – C6)

Reverse transcription was performed as per protocol using 2µl of RNA from all samples including standard and controls and PCR was performed testing standards and controls. PCR was repeated with the samples in conjunction with the standards and controls. PCR was repeated using the set protocol.

Constituents and volumes:

3µl forward primer (10µM)

3µl reverse (10µM)

3µl probe (5µM)

50µl universal mix

Then made up to 100 µl with ultra pure water and cDNA.

All volumes are for sample number, n =1. With increasing sample number the measurements need to be multiplied appropriately. 96µl of the mastemix were dispensed for each sample and 4µl of cDNA from each sample added correspondingly. The components were vortexed, spun down and 45µl evenly distributed in duplicates into wells of a sterile microtitre plate. The assay for GAPDH was mixed and dispensed in the same way. The real-time PCR programme was then run in the 7700 or 7500 ABI machines.

4.1.11 PCR for MMP2 Expression

Reverse transcription and real time PCR were performed similarly to the elastin RNA assay for the same samples. Probes from Eurogentec were used.

Sequences written 5' to 3'.

Forward primer HMMP2F:

GAG-ATC-TGC-AAA-CAG-GAC-ATT-GTA-TT

Reverse primer HMMP2R:

CCA-AAT-GAA-CCG-GTC-CTT-GA

Probe HMMP2T:

CAC-CAC-GGA-TCT-GAG-CGA-TGC-CAT

Dilutions were made for primer and probe with the primer amount of 10μM and of the probe 5μM. RT and PCR were performed after making standards and controls for n=50 (49 samples + 1).

Homo Sapiens MMP2 mRNA Genome

ORIGIN

```
   1 acatctggcg gctgccctcc cttgtttccg ctgcatccag acttcctcag gcggtggctg
  61 gaggctgcgc atctggggct ttaaacatac aaagggattg ccaggacctg cggcggcggc
 121 ggcggcggcg ggggctgggg cgcgggggcc ggaccatgag ccgctgagcc gggcaaaccc
 181 caggccaccg agccagcgga ccctcggagc gcagccctgc gccgcggagc aggctccaac
 241 caggcggcga ggcggccaca cgcaccgagc cagcgacccc cgggcgacgc gcggggccag
 301 ggagcgctac gatggaggcg ctaatggccc ggggcgcgct cacgggtccc ctgagggcgc
 361 tctgtctcct gggctgcctg ctgagccacg ccgccgccgc gccgtcgccc atcatcaagt
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 661 ccaagtggga caagaaccag atcacataca ggatcattgg ctacacacct gatctggacc
 721 cagagacagt ggatgatgcc tttgctcgtg ccttccaagt ctggagcgat gtgaccccac
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1321 tgtccactgt tggtgggaac tcagaaggtg ccccctgtgt cttccccttc actttcctgg
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1921 ggaatgaata ctggatctac tcagccagca ccctggagcg agggtacccc aagccactga
1981 ccagcctggg actgccccct gatgtccagc gagtggatgc cgcctttaac tggagcaaaa
2041 acaagaagac atacatcttt gctggagaca aattctggag atacaatgag gtgaagaaga
2101 aaatggatcc tggcttcccc aagctcatcg cagatgcctg gaatgccatc cccgataacc
2161 tggatgccgt cgtggacctg cagggcggcg gtcacagcta cttcttcaag ggtgcctatt
2221 acctgaagct ggagaaccaa agtctgaaga gcgtgaagtt tggaagcatc aaatccgact
2281 ggctaggctg ctgagctggc cctggctccc acaggccctt cctctccact gccttcgata
2341 caccgggcct ggagaactag agaaggaccc ggaggggcct ggcagccgtg ccttcagctc
2401 tacagctaat cagcattctc actcctacct ggtaatttaa gattccagag agtggctcct
2461 cccggtgccc aagaatagat gctgactgta ctcctcccag gcgccccttc cccctccaat
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2581 tttgggctgc cctggtgctg ccacacttca ggctcttctc ctttcacaac cttctgtggc
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2701 tggcacaggg cagtgggaca gggcatggcc aggtggccac tccagacccc tggcttttca
2761 ctgctggctg ccttagaacc tttcttacat tagcagtttg ctttgtatgc actttgtttt
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3001 tggcttcccg ctcagccctc cctgccctc ccttcaacca ttccccatgg gaaatgtcaa
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3481 ttaacctttc caaccacata aataaaaaag gccattatta gttgaaaaaa aaaaaaaaaa
3541 aaaaaaaaa
```

Figure 4.10 Homo sapiens matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase) (MMP2), transcript variant1, mRNA.VERSION NM_004530.4 GI: AUTHORS Menon,R., Pearce,B., Velez,D.R., Merialdi,M., Williams,S.M., Fortunato,S.J. and Thorsen,P

(Yellow – Forward Primer; Green - Reverse Primer; Red – Probe)

4.1.12 PCR Oestrogen Receptor alpha

Primers and Probe from Eurogentec were used. Sequences written 5' to 3'.

Forward primer HESR1F:

TGA-TGG-GCT-TAC-TGA-CCA-ACC-T

Reverse primer HESR1R:

CAA-AGC-CTG-GCA-CCC-TCT-T

Probe HESR1T:

AGT-TGA-TCA-TGT-GAA-CCA-GCT-CCC-TGT-CTG

The assay was set up and performed as the assays for elastin and MMP2. The expression of elastin, MMP2 and ERα were measured relative to the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) to normalise the data to amount of RNA extracted and cDNA produced from any preparation.

The primers and probe for GAPDH were

Human GAPDH probe

TGG-AGT-CCA-CTG-GCG-TCT-TCA-CCA

Forward primer

ATG-CTG-GCG-CTG-AGT-ACG-T

Reverse primer

AGC-CCC-AGC-CTT-CTC-CAT

Similarly, collagen XVII expression was measured by reverse transcription and real-time PCR. As the collagen XVII assay is less standard as the previous 2 assays some details are given again – see below.

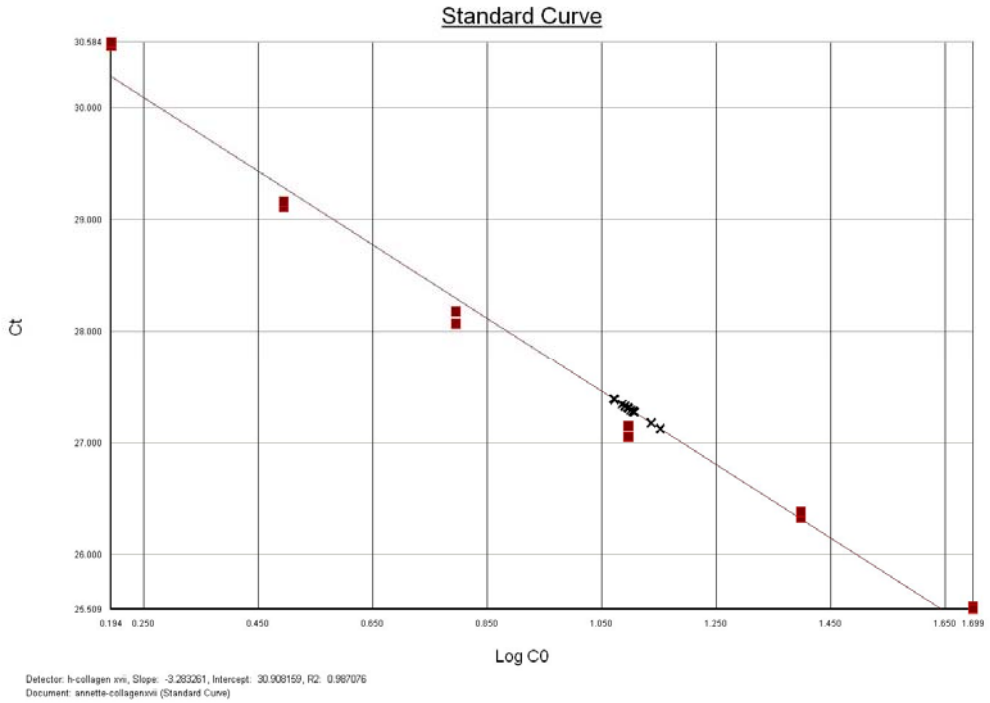

Figure 4.11 Logarithmic Standard Curve Graph for Collagen XVII

Homo Sapiens Collagen, Type XVII, alpha 1 (COL17A1), mRNA

ORIGIN

```
       1 aacatttttg aaagatcact cagctttaac acaccttggc tgggtctgga taaaaaaaaa
      61 gtgagcactg caaatttcta gaagaaaaca tcaggagaag aaagagagag ggggatttat
     121 tcaaagttgt ttccaattcc ttcaaaacct caaaccaggt ggctatggta tggatgtaac
     181 caagaaaaac aaacgagatg gaactgaagt cactgagaga attgtcactg aaacagtaac
     241 cacaagactt acatccttac caccaaaagg cgggaccagc aatggctatg ctaaaacagc
     301 ctctcttggt ggagggagcc ggctggagaa acaaagcctg actcatggca gcagcggcta
     361 cataaactca actggaagca cacgaggcca tgcctccacc tctagttaca ggagggctca
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     541 ggaatttgca tcttcttcaa ccagaggacg gagtcaaaca cgagagagtg aaattcgagt
     601 tcgactgcag agtgcgtccc catccacccg atggacagaa ttggatgatg ttaagcgttt
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     721 catccccaag aaaggcactg tggagaccaa aattgtgaca gcgagctccc agtcggtgtc
     781 aggcacctac gatgcaacga tcctggatgc caaccttccc tcccatgtgt ggtcctccac
     841 cctgcccgcg gggtcctcca tggggaccta tcacaacaac atgacaaccc agagctcatc
     901 cctcctcaac accaatgcct actctgcggg atcagtcttc ggagttccaa acaacatggc
     961 gtcctgctca cccactttgc accctggact cagcacatcc tcctcagtgt ttggcatgca
    1021 gaacaatctg gcccccagct tgaccaccct gtcccatggc accaccacca cttccacagc
    1081 atatggggtg aagaaaaaca tgccccagag tcctgcggct gtgaacactg gcgtttccac
    1141 ctccgccgcc tgcaccacaa gtgtgcagag cgatgacctt ttgcacaagg actgcaagtt
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    1261 caaggacagc gggaaggtct ttacagcctc ccctgccagc atcgctgcaa cttctttttc
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       ...
```

Figure 4.12 Sequence of Homo Sapiens Collagen XVII mRNA with Primer and Probe. Accession number NM_000494 from NCBI

http://www.ncbi.nlm.nih.go

(Yellow - forward primer, Green - reverse primer, Red – probe)

Chapter 5 :

Results

5.1 Racial study

Demographics for black and white women in the stress incontinence were assessed. Paraffin embedded vaginal skin tissue was collected from 46 women attending Tygerberg hospital, Cape Town. 36 women were premenopausal and 10 menopausal (4 of the latter were taking HRT). As initial analyses showed secondary effects with HRT (see below), the comparison of black and white women and effects of stress incontinence were analysed in the premenopausal women alone.

Four groups were established:

white controls (WC),
white stress incontinent (WS),
black controls (BC)
black stress incontinent (BS)

There were 3 women who smoked in the WC and WS groups, one woman smoked in the BC group while none of the women in the BS group were smokers. However, no secondary effects for smoking were shown. Also, no secondary effects for parity or age on elastin staining for these women were shown.

Summary of Demographics Stress Incontinence Study

Group	n	Age mean(range)	Premenopausal	Parity mean(range)
WC	10	39 (20-54)	all	2.1 (1 -4)
WS	7	41 (34 – 47)	all	2.6 (2-4)
BC	11	34 (23 – 42)	all	2 (0-4)
BS	8	43 (35 – 59)	all	3.4 (2 -5)

Table 5.1 Patients' Details for Elastin Protein Content in Stress Incontinence in Different Races Study.

The samples were assessed for percentage of elastin protein content using histochemistry staining as described earlier.

Before the below analyses were carried out with the premenopausal women only, a comparison of the postmenopausal women with the premenopausal women was performed. 4 of the postmenopausal women in the WS group were taking HRT. Comparing women taking HRT within the WS group with those that were not showed that those women taking HRT had significantly raised elastin staining. Using unpaired 't' test within the WS group, no HRT mean +/- SEM was 12.6 +/- 0.97 (n=9) while with HRT mean +/- SEM was 18.2 +/- 1.63 (n=4), $p<0.05$. This suggested that HRT treatment might be having an effect on elastin expression.

Results from the histochemistry measurements from the premenopausal women alone were therefore analysed using ANOVA (Analysis of variance). ANOVA provides a statistical calculation to assess if the means of several groups are all equal. It therefore generalizes Student's two-sample t-test to more than two groups.

The percentage elastin and collagen I and collagen III content was measured using computer-assisted image analysis (Zeiss KS400 software, Image Associates, Bicester, UK) to evaluate histochemical and immunocytochemical staining.

For all graphs in both studies:
p value (probability) = significance, <0.01 (highly significant), <0.001 (very highly significant)
SEM = standard error of the mean

Percentage Elastin Content

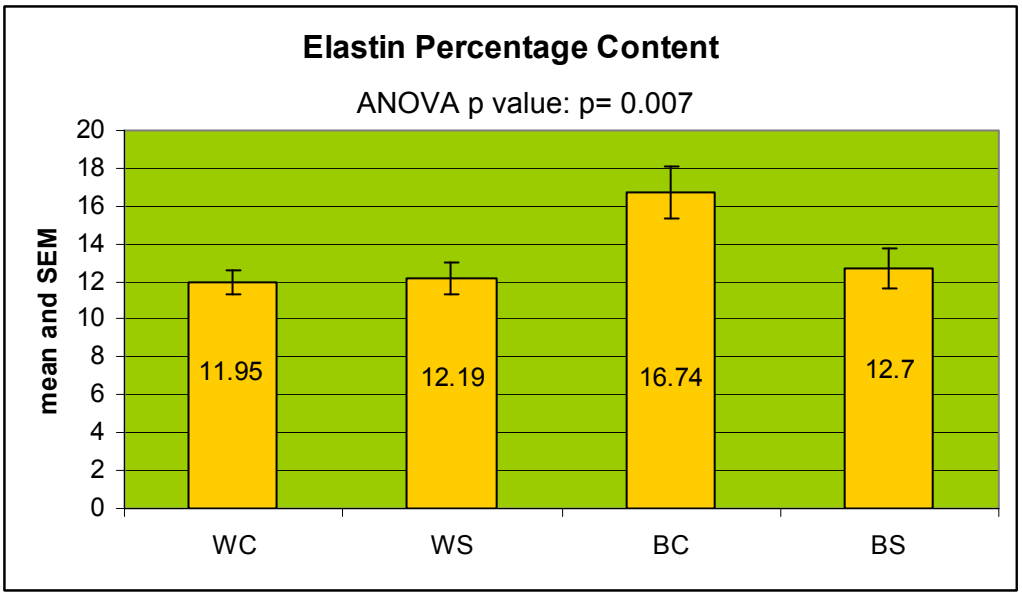

Figure 5.1 Results for Elastin Protein Content (Histochemistry)

ANOVA comparison of the means of the different groups. (mean +/- SEM; BC 16.74 +/- 1.26, BS 12.70+/- 1.00, WC 11.95 +/- 0.63, WS 12.19+/- 0.91)
The ANOVA p value was p= 0.007

Black women have a higher elastin protein content which decreases significantly if an insult to the skin such as stress incontinence has happened. Meanwhile white women have a lower elastin content compared with black women but white women with SI are not significantly different from those without SI. These specific comparisons are shown below.

Elastin Protein Content in BC compared to WC

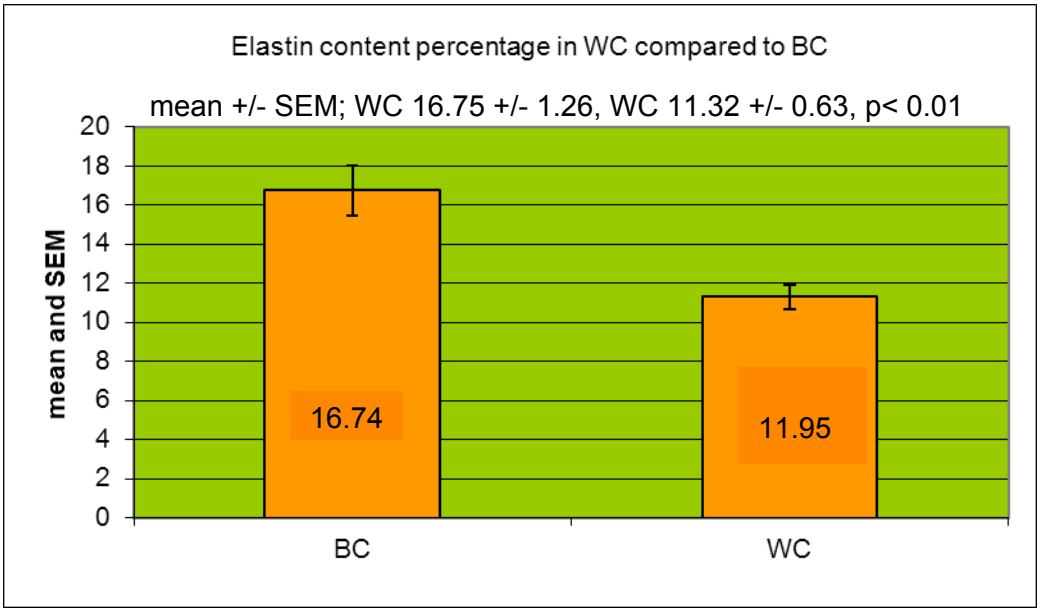

Figure 5.2 Elastin protein content percentage in WC compared to BC

Unpaired t-Test was used to compare the means in the two goups. (mean +/- SEM; WC 16.75 +/- 1.26, WC 11.32 +/- 0.63, p< 0.01).

Black women without stress incontinence have a higher elastin content in periurethral vaginal skin compared to white women without stress incontinence.

Elastin Protein Content in BC compared to BS

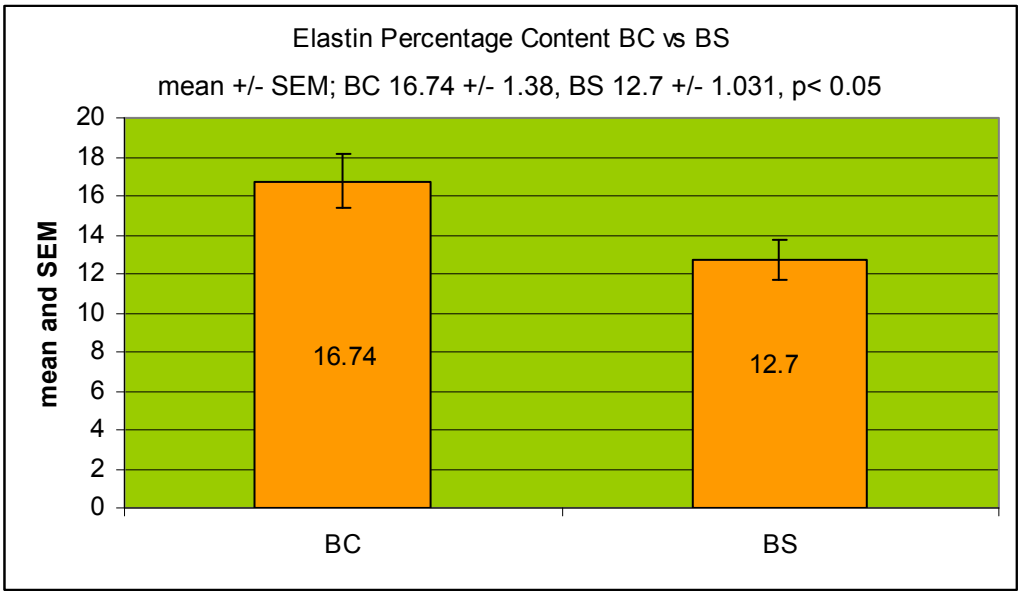

Figure 5.3 Elastin protein content percentage in BC compared to BC

Unpaired student-t- test was used to compare the means in the two goups. (mean +/- SEM; BC 16.74 +/- 1.38, BS 12.7 +/- 1.031, p< 0.05)

Interestingly, black women with SI showed significantly lower elastin protein content compared to peers with no SI.

Examples of the elastin staining in the 4 different groups are shown below.

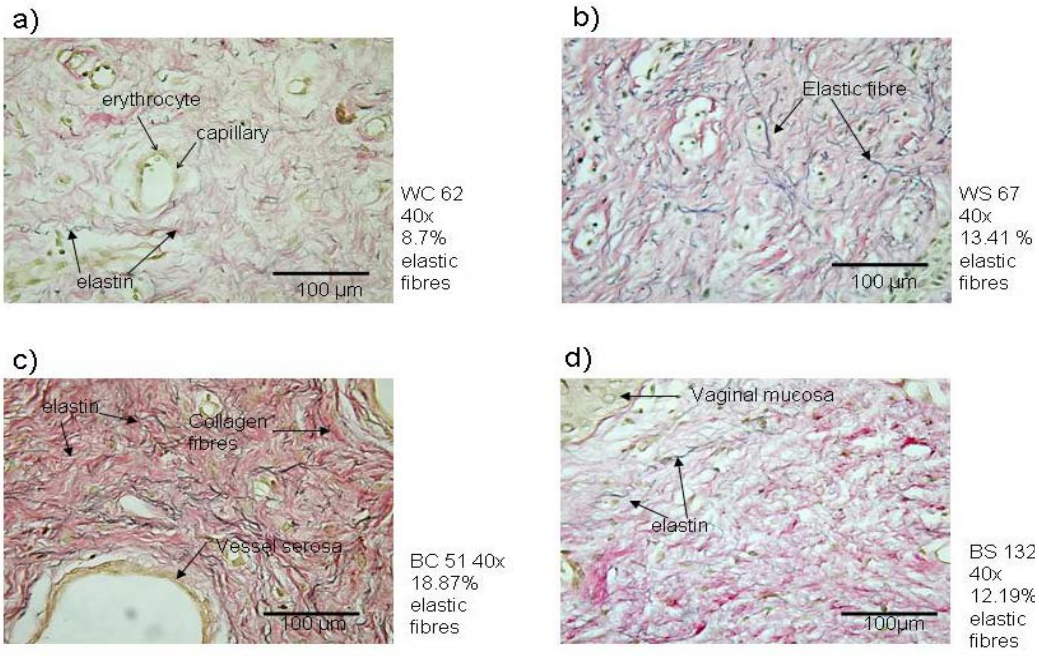

Figure 5.4 WC, WS, BC and BS percentage elastin content at 40 x magnification in comparison. Blood vessels, collagen and elastin fibres are indicated with arrows.

5.2 Results Prolapse Study

Samples from 16 women before and after treatment were assessed for the prolapse study. The extracellular matrix components in the vaginal tissue before and after three months treatment with Vagifem® were examined.

The age ranged between 47-84 years with a mean of 59. 14 women were non-smokers, 2 smoked between 5-10 cigarettes per day. One lady stopped smoking whilst being on the trial. Parity ranged between 2 to 4, with a mean of 2.5. The majority of women had spontaneous vaginal deliveries, there were no reported Caesarean sections and only two women had instrumental deliveries. Both were forceps deliveries. The women's BMI varied between 23 to 31, with a mean of 27.6 and was calculated using the regular formula:

$$\text{BMI} = \frac{\text{weight (kg)}}{\text{height}^2\ (\text{m}^2)}$$

Because of the small subgroups it was not possible to show any secondary effects with the above variables.

The percentage elastin and collagen I and collagen III content was measured using computer-assisted image analysis (Zeiss KS400 software, Image Associates, Bicester, UK) to evaluate histochemical and immunocytochemical staining.

RNA was extracted using Trizol reagent and mRNA expression of each gene was measured relative to GAPDH using real time PCR for expression of MMP2, ERα, elastin and collagen XVII.

5.2.1 Molecular Biology Results

MMP2 Expression

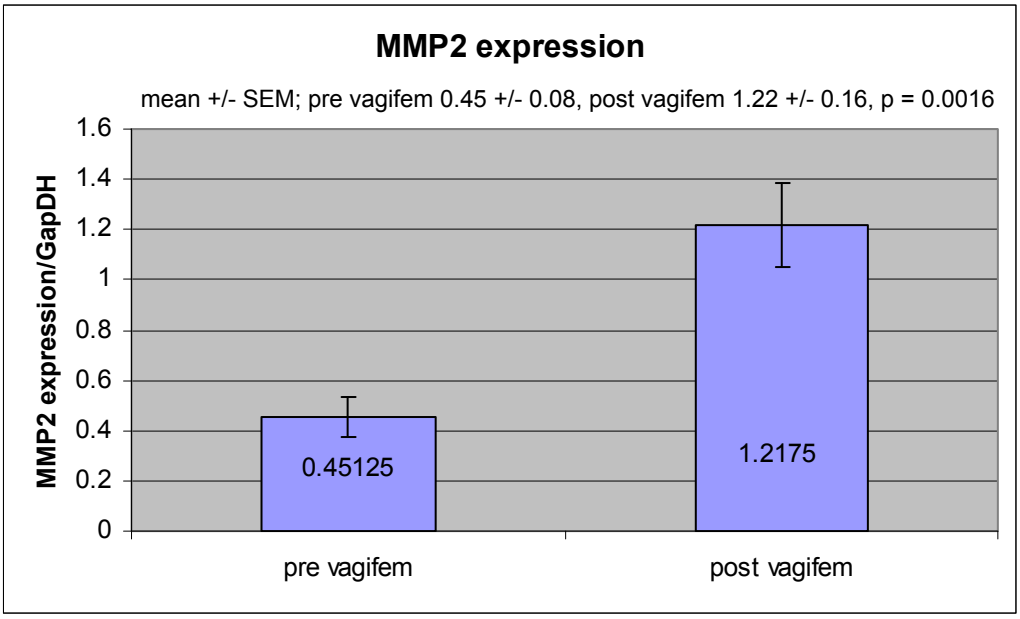

Figure 5.5 MMP2 RNA expression in relation to GAPDH, before and after Vagifem application, prolapse study

Sample groups were divided into pre and post vagifem treatment groups. Paired student t-test was performed to compare the means in these groups. (mean +/- SEM; pre vagifem 0.45 +/- 0.08, post vagifem 1.22 +/- 0.16, p = 0.0016.)

The MMP2 expression was highly significantly up-regulated after the use of Vagifem in women with POP.

Elastin mRNA Expression

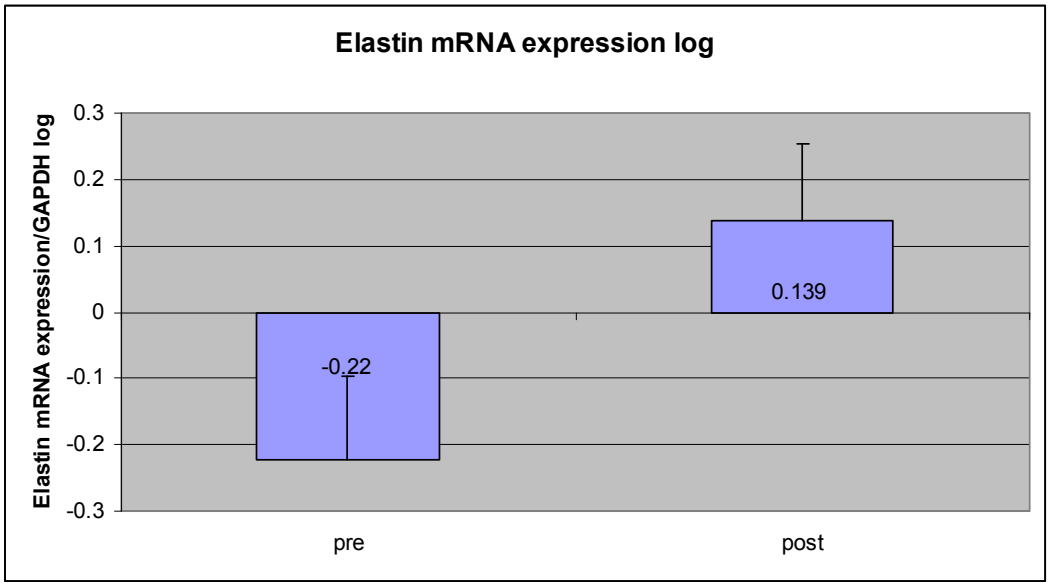

Figure 5.6 Elastin mRNA Expression in Relation to GAPDH Expression in logarithmic scale before and after Vagifem Application

The measured RNA expression for Elastin in the two groups was normalised with GAPDH, the results logarithmically transformed and Paired Student t-test was used to analyze and compare the means. (mean +/- SEM; pre vagifem -0.22 +/- 0.13, post vagifem 0.14 +/- 0.11, p-value see anti-logarithmic data).

Anti – logarithmic transformation of this data yields a pre-treatment mean and SEM range of 0.6 (0.45 – 0.8) and post treatment mean and SEM range of 1.38 (0.32 – 1.06) (see below).

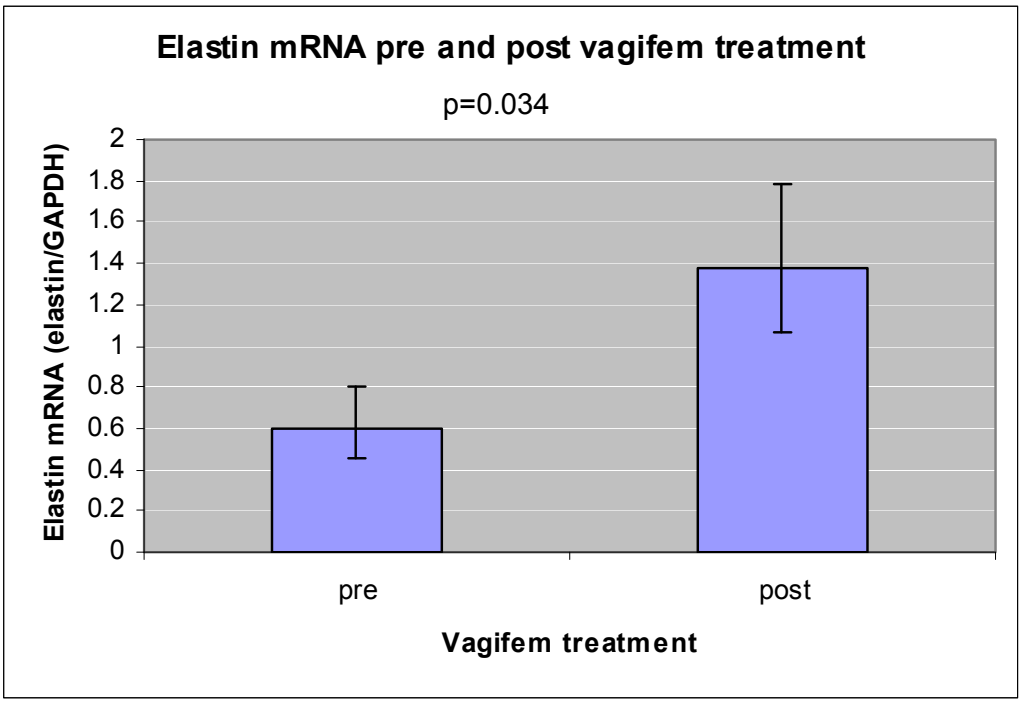

Figure 5.7 Representation of elastin mRNA expression after the logarithmic transformation. Post vagifem treatment elastin mRNA expression is significantly higher compared to pre- vagifem treatment (p=0.034).

Collagen XVII RNA Expression

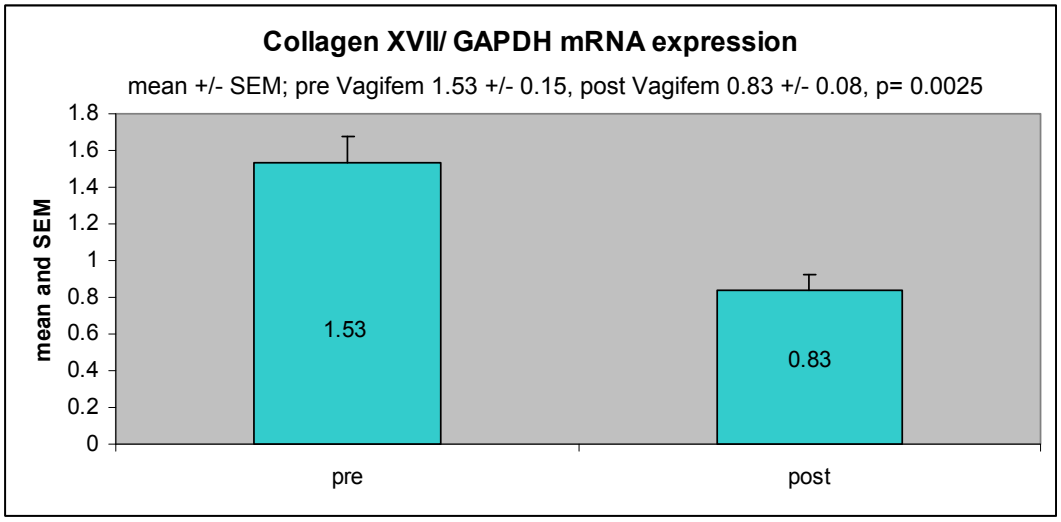

Figure 5.8 Collagen XVII mRNA Expression in Relation to GAPDH Expression

The measured mRNA expression for Collagen XVII in the two groups was also normalised with GAPDH. Paired Student's t-test was used to analyze and compare the means. (mean +/- SEM; pre Vagifem 1.53 +/- 0.15, post Vagifem 0.83 +/- 0.08, p= 0.0025.)

Collagen XVII was significantly decreased after three months of application with Vagifem. This process appears to show remodelling of the ECM as this result suggests reduced adhesiveness under the influence of locally applied oestradiol at this stage.

Oestrogen Receptor α mRNA Expression

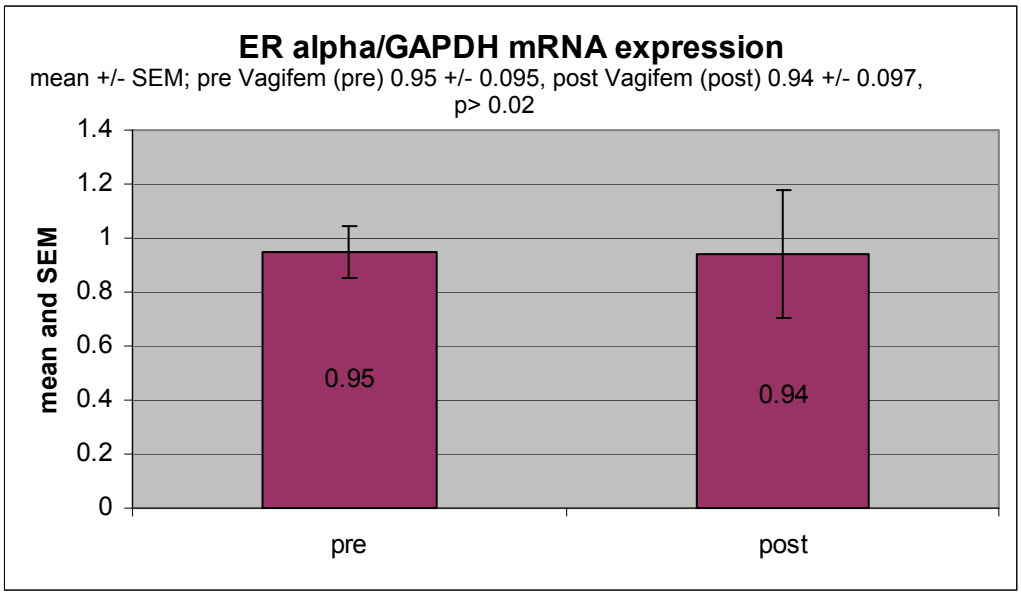

Figure 5.9 Oestrogen Receptor alpha mRNA Expression in Relation to GAPDH Expression

The measured RNA expression for ERα in the two groups was normalised with GAPDH. Paired Student t-test was performed to analyse and compare the means. (mean +/- SEM; pre Vagifem (pre) 0.95 +/- 0.095, post Vagifem (post) 0.94 +/- 0.097, p> 0.02) The p value is 0.93 and not considered significant.

5.2.2 Histochemstry and Immunochemistry Results

Elastin protein content in percentage

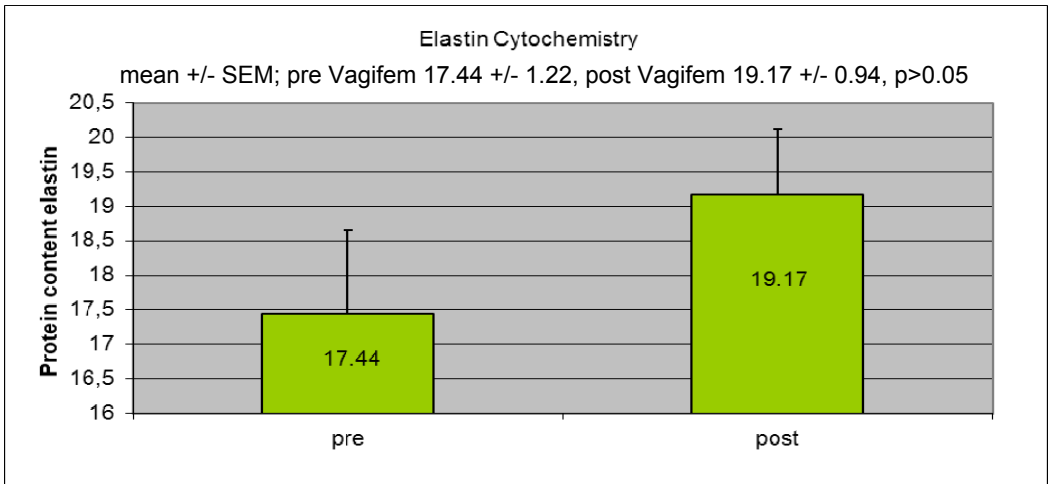

Figure 5.10 Elastin histochemistry results in percentage staining content, before and after Vagifem application

Elastin percentage content was measured using histochemistry techniques. The means of the two groups were analysed using paired student t-test. (mean +/- SEM; pre Vagifem 17.44 +/- 1.22, post Vagifem 19.17 +/- 0.94, p>0.05) the p value is 0.27 and not considered significant.

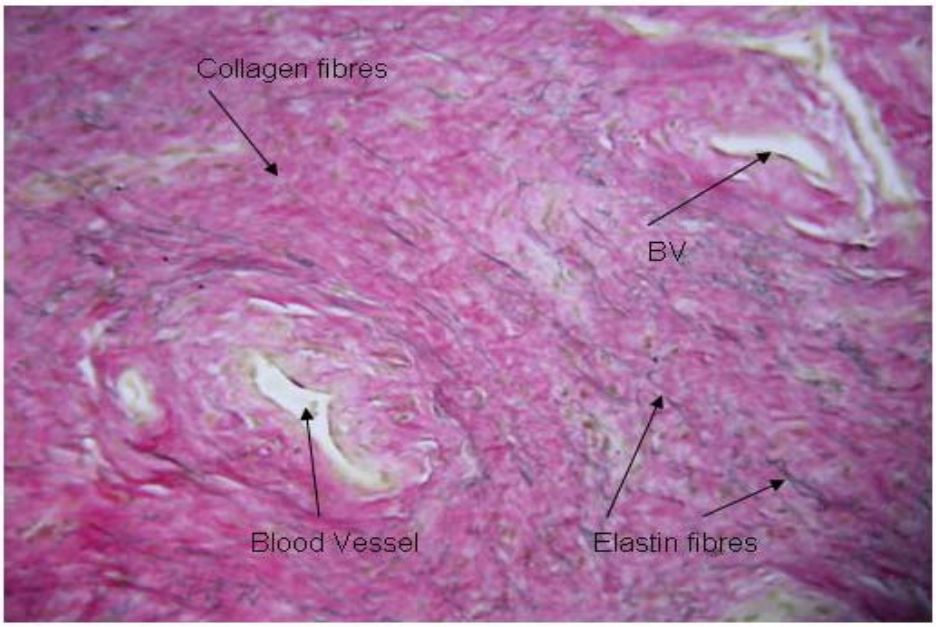

Figure 5.11 Example for Elastin percentage content prior to Vagifem application. Picture at 20x magnification. Elastin fibres appear black meanwhile collagen fibres are red. Blood vessels can be clearly seen.

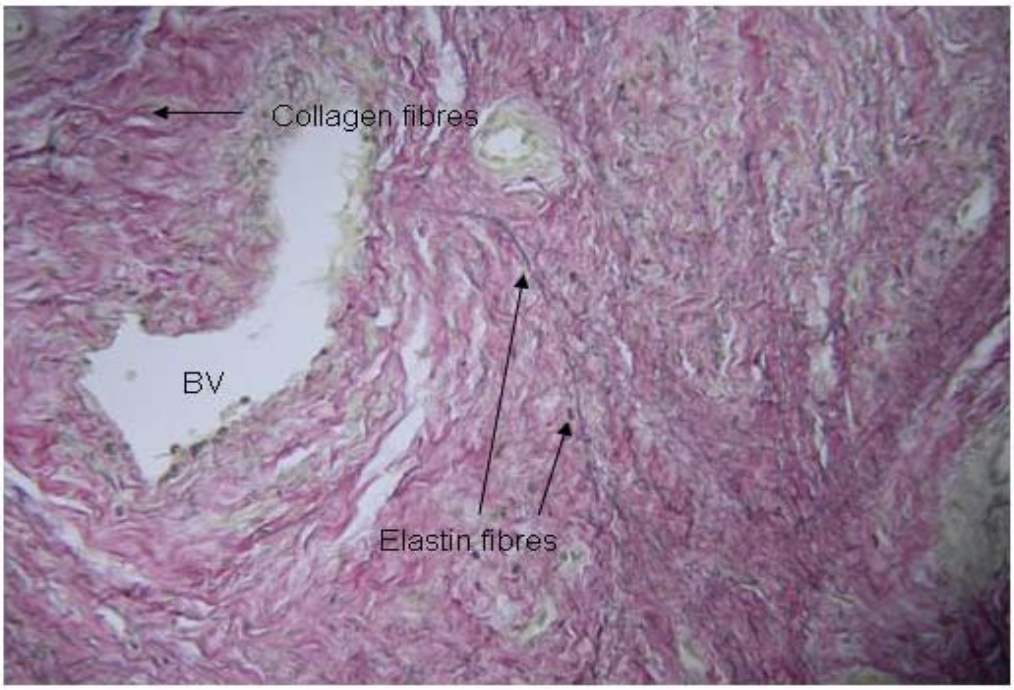

Figure 5.12 Example for Elastin percentage content after Vagifem application. Picture at 20x magnification. Elastin fibres appear black and collagen fibres red. There is an increase in elastin content compared to figure 5.11

Collagen I Protein Content in Percentage, Immunocytochemistry Results

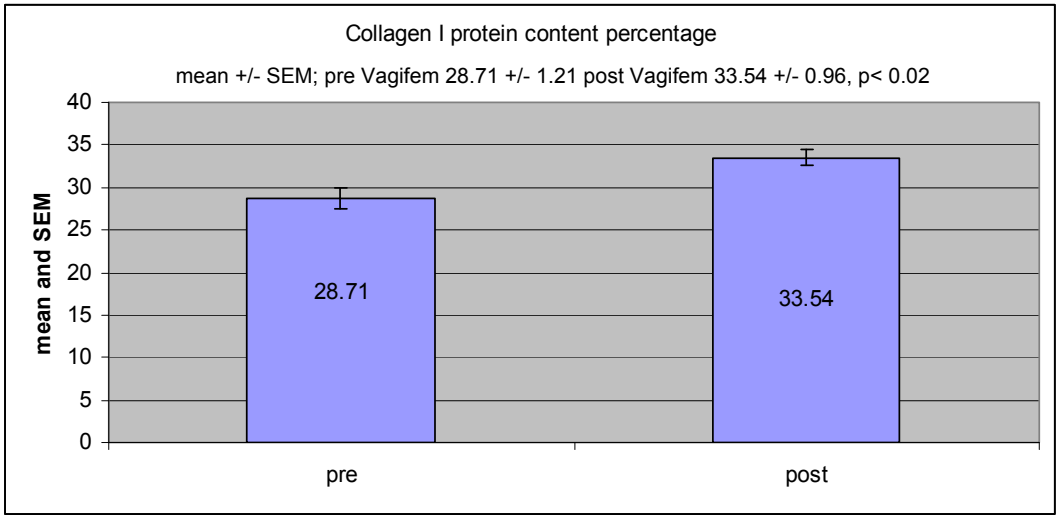

Figure 5.13 Collagen I Protein Content in Percentage

The percentage content of collagen I in the two groups was, as the elastin content, measured with Zeiss system analysis. Paired Student t-test was used to analyse and compare the means. (mean +/- SEM; pre Vagifem (pre) 28.71 +/- 1.21 post Vagifem (post) 33.54 +/- 0.96, p< 0.02) The p value was 0.0003.

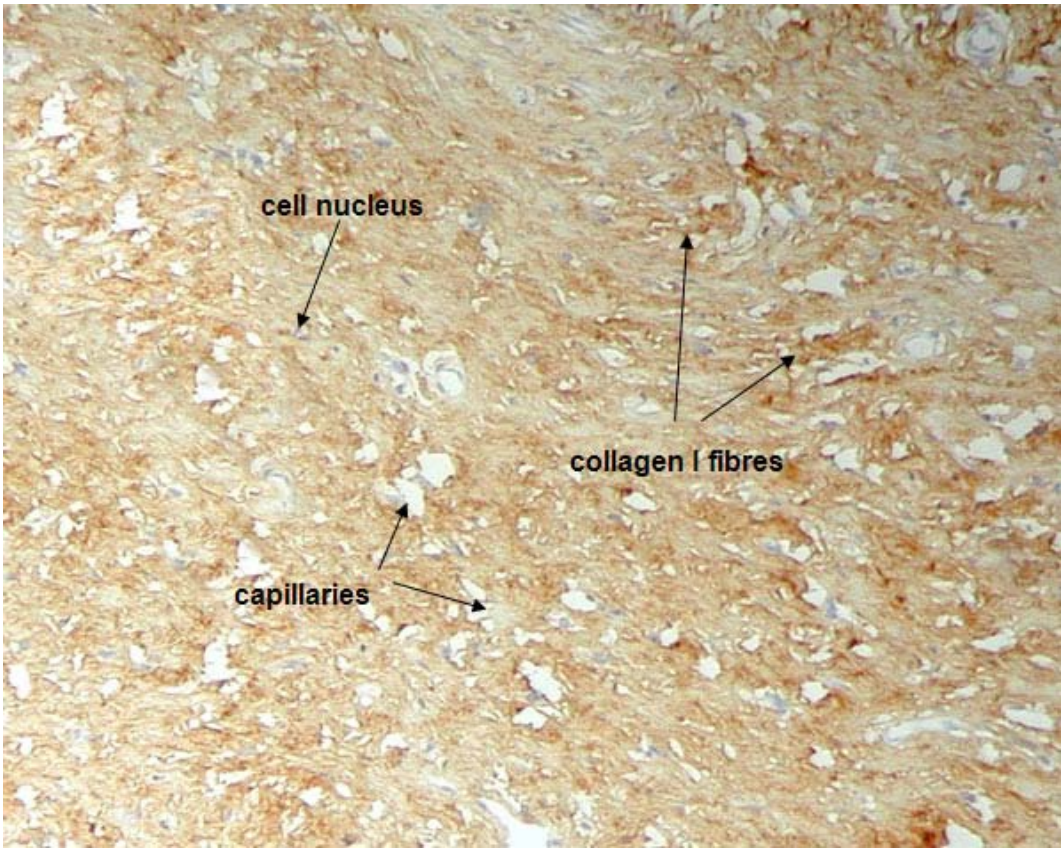

Figure 5.14 Collagen I fibres before treatment with Vagifem. The fibres appear brown and are surrounded by blood vessels and capillaries. Purple black cell nuclei are visible in this x20 magnification.

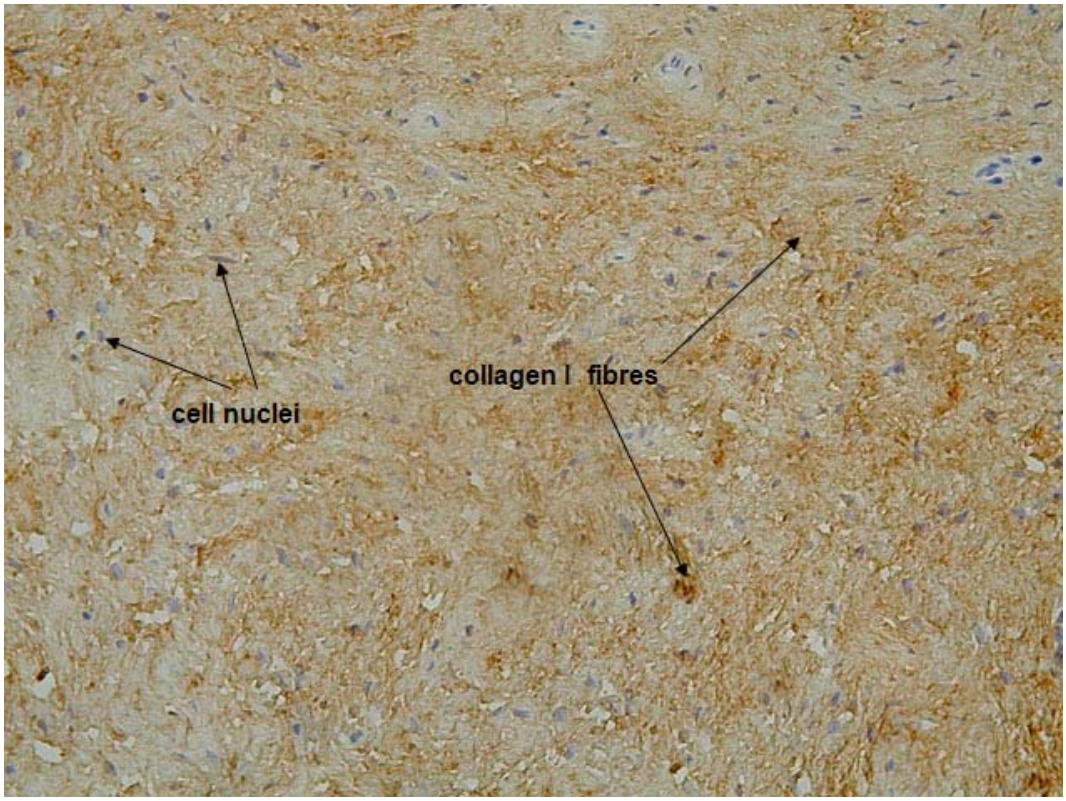

Figure 5.15 Collagen I fibres in x20 magnification from vaginal wall tissue after Vagifem application. In comparison to Figure 5.15, collagen fibres appear more dense.

Collagen III Protein Content in Percentage

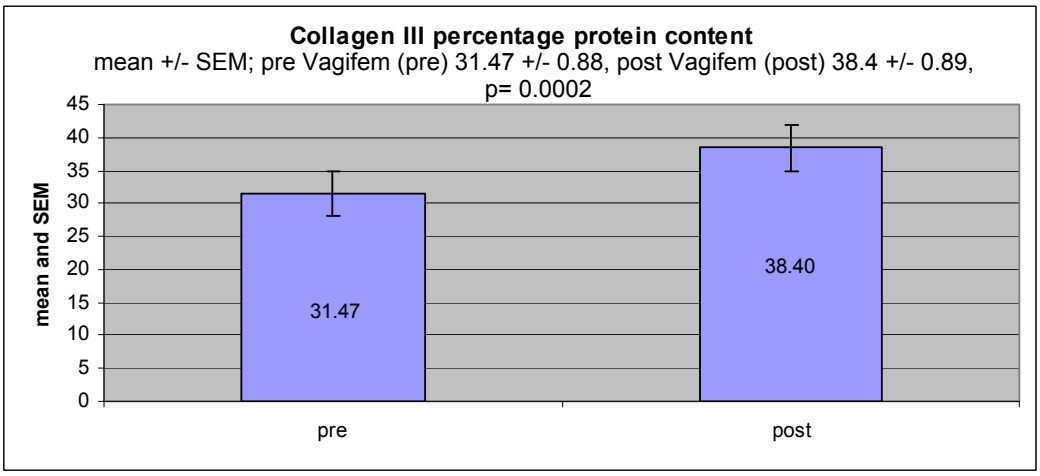

Figure 5.16 mean and SEM of Collagen III protein, pre and post Vagifem application.

The collagen III percentage protein content in the two groups was measured with Zeiss system analysis. Paired Student t-test was used to analyse and compare the means. (mean +/- SEM; pre Vagifem (pre) 31.47 +/- 0.88, post Vagifem (post) 38.4 +/- 0.89, p= 0.0002.)

Collagen III was found to be increased significantly after Vagifem application similar to collagen I. This finding complements the elastin results and demonstrates increased elasticity to support the prolapsed tissue and prevent further damage.

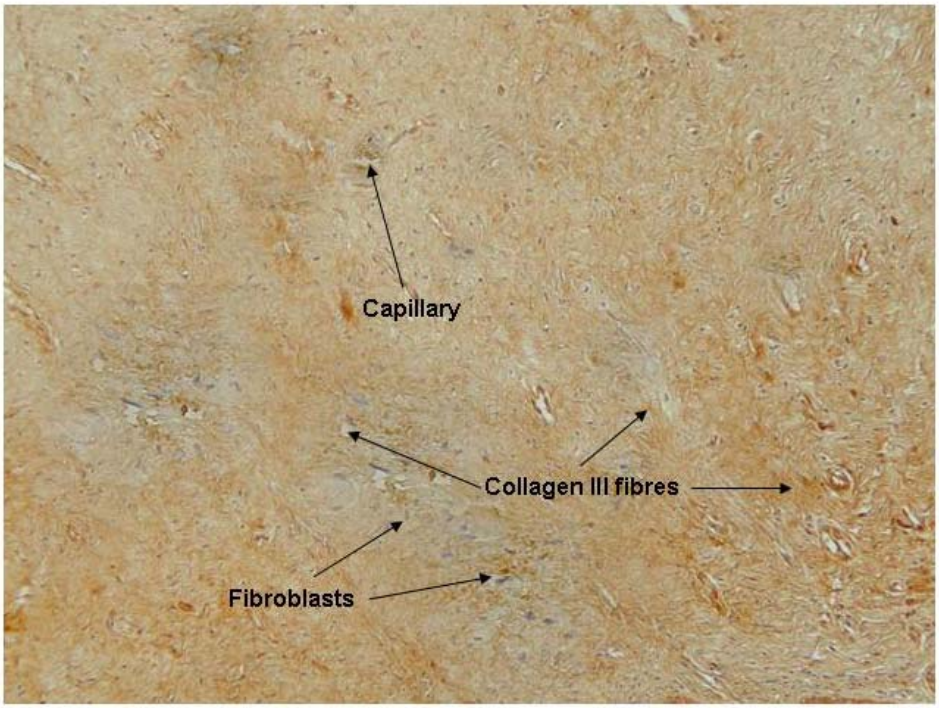

Figure 5.17 Collagen III staining of vaginal skin in a woman with POP prior to Vagifem application. Collagen III stains in brown. Fibroblasts are also identified. Microscope amplification used for this picture was 20X.

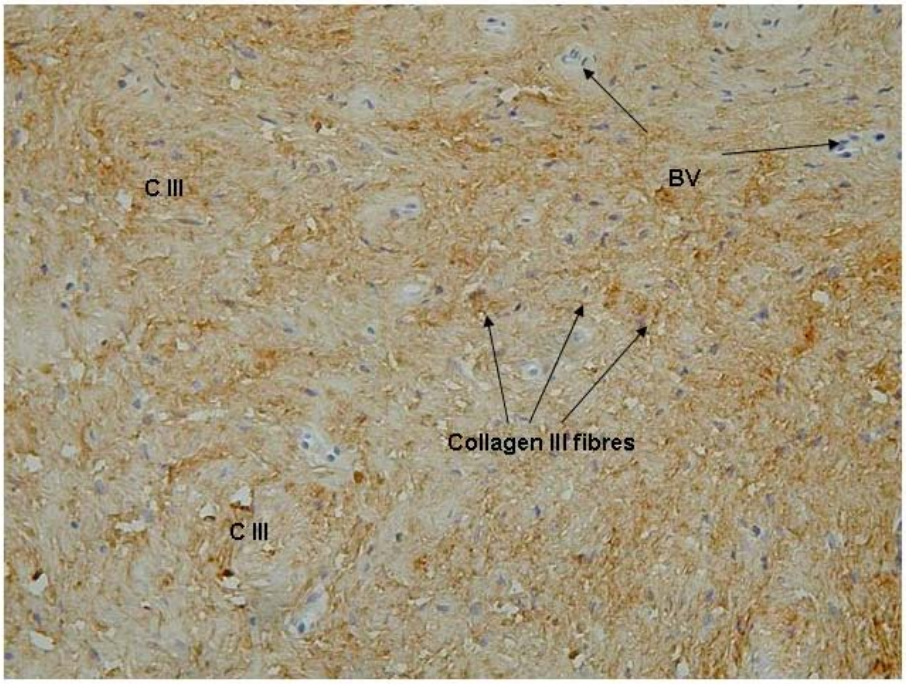

Figure 5.18 Collagen III (C III) staining of vaginal skin from same woman as seen in Figure 5.17 after Vagifem application. Dense collagen III formation can be seen with this staining. Blood vessels (BV) are seen in the connective tissue. Microscope amplification is x20

Collagen I:III Ratio Protein Content Percentage

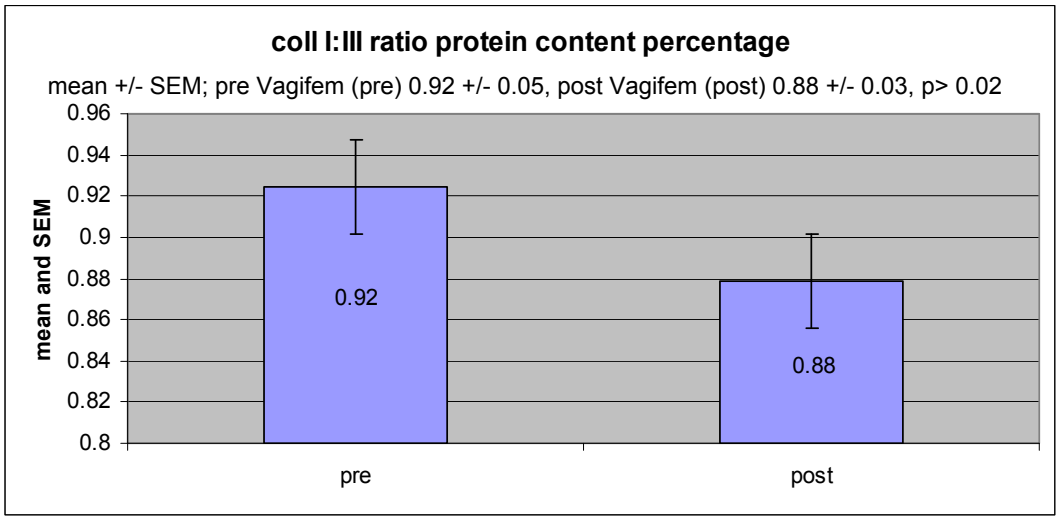

Figure 5.19 mean and SEM of Collagen I:III ratio, pre and post Vagifem application.

The percentage protein content of collagen I in relation to percentage protein content of collagen III in the two groups was measured with Zeiss system analysis. Paired Student t-test was used to analyse and compare the means. (mean +/- SEM; pre Vagifem (pre) 0.92 +/- 0.05, post Vagifem (post) 0.88 +/- 0.03, p> 0.02) The p value is 0.41 and not considered significant.

Interestingly, both Collagen I and Collagen III content increased under the influence of Vagifem and the collagen I:III ratio was not changed significantly.

Results overview

	Pre mean (+/- SEM)	Post mean (+/- SEM)	P value	n
MMP2 mRNA	0.45 (0.078)	1.22 (0.16)	0.0016**	16
Elastin mRNA (anti log)	0.6 (0.45-0.8)	1.38 (0.32-1.06)	0.034	16
Elastin protein	17.44 (1.22)	19.17 (0.94)	0.27	16
CollagenXVII mRNA	1.53 (0.15)	0.83 (0.08)	0.0025**	16
ERalpha mRNA	0.95 (0.095)	0.94 (0.097)	0.93	16
Collagen I protein	28.71 (1.21)	33.54 (0.96)	0.0003***	16
Collagen III protein	31.47 (0.88)	38.4 (0.89)	0.0002***	16
Collagen I:III ratio	0.92 (0.05)	0.88 (0.03)	0.41	16

Table 5.2 Result Overview for the Prolapse Study

5.3 Summary

5.3.1 Racial and Stress Incontinence Study

It was established that black women have a higher elastin content in their periurethral vaginal tissue than white women. As predicted black women with stress incontinence had a lower elastin level than black women without SI. However, the connective tissue of white women appeared to respond differently than that of black woman as white women with stress incontinence did not have significantly different levels of elastin staining compared to white controls. However, white women can have secondary effects as postmenopausal women on HRT appeared to have higher elastin levels but if this was an oestrogen effect, the following Vagifem study was necessary to prove this. What secondary effects were occurring in the premenopausal white women was not established. However, it could be that black women have the possibility to withstand pelvic floor dysfunction more successfully because of their higher elastin levels.

Increased elasticity would support the tissue and induce more resilience to changes due to stress to the tissue.

5.3.2 Prolapse Study

We could show that locally applied oestrogen up-regulates the message (mRNA) for MMP2 necessary for tissue remodelling after stress. Although the message for ERα was found in vaginal skin and therefore had the potential to respond to topical oestrogen ERα mRNA remained unchanged after Vagifem administration, contrary to original predictions. The message for the adhesive collagen, collagen XVII, was down-regulated significantly probably contributing to tissue remodelling. However, collagen I protein staining was increased significantly, suggesting new synthesis. Interestingly, mRNA for elastin was also significantly elevated after 3 months of Vagifem treatment but the increase in protein elastin staining did not quite reach significance. Overall this small trial provides some evidence that topical oestrogen has beneficial effects on message and protein production of key factors involved in tissue remodelling to improve vaginal tissue characteristics.

Chapter 6 :

Discussion

Our hypothesis that black women would express higher elastin levels in vaginal tissue than white women has been found to be correct. This is an original finding and supports results from a previous study in this research group which saw lower collagen I in respect to collagen III in black women compared with white. Together, results indicate higher elasticity in vaginal tissue of black women. This hypothesis was put forward as black women have been considered to be more resistant to stress incontinence and vaginal prolapse. An increased elasticity of the tissue would enable it to compensate more appropriately when there is stress on the tissue.

As also discussed in the Introduction, previous studies have demonstrated that black women have a lower prevalence of pelvic floor dysfunction such as stress incontinence or POP.[148] Some inherent anatomical and physiological differences have been widely established in the past. For instance, in these studies black women were shown to have a higher bone mass density and muscle mass and have a lower incidence of bone fracture and trauma to the connective tissues. [149] Bone remodelling appears to be different and together with a modified calcium metabolism osteoporotic fractures are less common among black women. Studies using MRI established that black women have a larger levator ani volume and a closer attachment of the puborectalis muscle compared to white women. The different muscle volume and position resulted in a variation of bladder neck position, urethral angle and pubic arch angle. Other studies demonstrated a longer urethra and higher urethral closure pressure. The increased elasticity of the vaginal tissue would thus add to a list of beneficial adaptations that are present in and around the vagina of black women to help them resist stress incontinence and vaginal prolapse. Fitting with the above advantages pelvic ligaments of black women with prolapse show greater collagen content compared to white peers[150] and black women tend to have a lower incidence of perineal

tears during vaginal birth, demonstrating higher tissue resilience.[151] Interestingly, black women also tend to have a higher incidence of uterine fibroids, keloid scars and striae[33] than white women supporting the presence of a different skin extracellular matrix make-up in black women.

The next hypothesis in this thesis was that in stress incontinent women elastin content would change in reaction to stress. This was indeed confirmed in black women where stress incontinence coincided with significantly lower elastin levels than control. However, elastin content remained unaltered in white SI or control women. We currently don't have a specific explanation for this but confounding factors or secondary effects should be considered.

Chen et al examined periurethral vaginal wall fibroblasts from premenopausal women with stress urinary incontinence and compared these to fibroblasts form continent women. Total elastase activity was measured by generation of free amino groups from succinylated elastin. It appeared that elastase activity was increased in relaxin-stimulated stress urinary incontinence fibroblasts by either decreased inhibitor (alpha-1 antitrypsin) production or increased elastase activity[127]. Another study by Chen showed a significant decrease in alpha-1 antitrypsin expression in tissues from women with SUI/POP compared to controls. Collectively, data suggests that altered elastin metabolism may contribute to the connective tissue alterations observed in pelvic floor dysfunction.[152] This increase in elastase could explain the decrease in elastin in black incontinent women but for the case of white women with stress incontinence perhaps other factors inhibit the elastase activity.

Laborda et al demonstrated that Collagen XVII which is an adhesive molecule contributing to tensile strength was expressed in higher quantities (mRNA measurements) in the vaginal wall skin of black women compared to white[36]. These latter findings proposed that black women have elevated adhesiveness. Therefore it was decided to examine elastin protein content in the vaginal skin of women from different races with and without stress incontinence to show improvements in elasticity as well.

Elastin is an amorphous protein and belongs to the group of elastic fibres including elaunin and oxytalan. They are produced by fibroblasts and smooth muscle cells in arteries. Extension can reach up to 1.5 times of the basic length, and recoil without major damage to the original length when relaxed. The elastic fibre consists of microfibrils which build a scaffold for the elastin protein. These microfibrils are assembled as numerous proteins such as microfibrillar-associated glycoproteins, fibrillin, fibullin.

The increased elastic property of the tissue in black women seems to protect the tissue from damage for a longer period of time but the remodelling process after damage occurred may be less active. Higher elastin content could be accountable for the reduced risk of perineal tears during vaginal child birth in black women but the tendency to have keloid scars could be a sign that the remodelling process of the tissues once damage is present is not effective anymore. Collagen III is inserted into the scar tissue in replacement of collagen I supposedly giving a higher elasticity of the tissue but without the vigour of normal scar tissue. The tissue appears to restructure itself and regain the previous quality of elasticity and strength but is not capable of achieving satisfactory results.

The main study population contained only pre-menopausal women hence excluding potential hormonal differences due to menopause. Hormonal influences are important confounders since in a small subgroup of postmenopausal women HRT caused an elevation of elastin levels in white incontinent women. Other parameters influencing extracellular matrix components such as smoking, BMI, diet and life style were examined similarly but no significant secondary effects were evident.

The second study in this thesis was designed after the first study showed that increased elasticity in black women may be a major reason for the lower prevalence of stress incontinence and prolapse in these women. Hormonal influences are also relevant as in a small group of postmenopausal women on HRT higher levels of elastin were observed. Therefore, it was hypothesised that in postmenopausal women with vaginal prolapse oestrogen (Vagifem) would increase elastin mRNA and protein levels.

In the present thesis study, women with at least POP stage 2 were recruited and ECM changes were assessed in the prolapsed vaginal wall tissue before and three months after application of Vagifem, containing 25mcgr 17β oestradiol. Three months of oestradiol treatment changed the expression and protein content of ECM components in the vaginal skin. Collagen I and III protein content in the vaginal skin increased both significantly leaving the collagen I:III ratio unaltered. Importantly elastin mRNA was found to be elevated three months after Vagifem treatment. Elastin protein level, although also higher after oestrogen treatment, did not quite reach significance. However, the study only lasted three months and one would anticipate that after six months the elastin staining would be more pronounced. Jackson et al studied administration of oestrogen for six months in postmenopausal women with stress incontinence and found new

collagen was being synthesised by then and also pro-MMP2 was up regulated to assist in tissue remodelling. This fits well with the up regulation of MMP2 mRNA levels after three months in the present work. The present study also showed a significant reduction in the adhesive collagen, collagen XVII. Presumably this would aid in remodelling, although this collagen would have to be up regulated eventually when the ECM had been remodelled adequately to establish tissue integrity.

To avoid confounding hormonal influences, only post-menopausal women were recruited for the study. A two year gap since last menstruation or the discontinuation of hormone replacement therapy of any kind for at least six months had to be ensured. As explained in the introduction, no hormonal supplemental medication should be used, obviously, yet HRT plays a large role in the management of postmenopausal symptoms and in the prevention of osteoporosis in postmenopausal women. It therefore proved to be quite difficult to recruit women fulfilling the criteria for this study. Also, many women were referred for recurrent prolapse repair surgery as the Princess Anne Hospital in Southampton is a tertiary unit. These patients also needed to be excluded. As also demonstrated in previous studies, many women presenting with POP also experience stress incontinence symptoms. Stress incontinence on its own can alter the pelvic floor and therefore extracellular matrix organisation. Patients with SI symptoms or urodynamically proven SI were not recruited.

One further impediment was the precaution of women using hormone supplements. The fear of side effects such as breast cancer prevented many women to take part in the study unless they were placed in the control group. Even detailed and reassuring counselling with the use of patient information leaflets did often not help to change patients' mind. As recruitment was difficult

for the above reasons the setting up of a control group was not easy and the manufacturers of Vagifem could not produce any placebo pessaries in time. Therefore, the patients had to act as their own control group of pre and post treatment. However, we considered the design still adequate since a previous study by Jackson had shown that the production of new collagen and pro-MMP2 was due to oestrogen treatment as the placebo control group that ran alongside the study group remained unchanged.

The aetiology of prolapse is still poorly understood. Theories of factors to increase the risk of developing POP include muscular or neurological injury especially occurring during child birth[153], obesity, smoking and family predisposition. Rahn et al showed in an animal study that matrix metalloprotease (MMP) activity is increased in the postpartum vagina of wild-type (WT) animals. This derivative activity is also accompanied by an increase of elastic fibre synthesis and assembly. [154]

Oestrogen plays an important role in maintenance of the pelvic floor tissues and appears to improve hydration and strength of skin. It was shown that a decrease in sex steroids induces a reduction of those skin functions that are under hormonal control. [155]As an opposite effect, topical oestrogen improves elasticity and collagen turnover.[156] Shah and Maibach showed that skin is thinning with age and that topical oestrogen is slowing down this effect. Further, DNA synthesis is stimulated in human epidermal keratinocytes by oestradiol which was shown by Urano et al in 1995. There are different effects of oestrogen via multiple oestrogen receptors both, intra cellular as the known steroid receptors (here ERα and ERβ) or as transmembrane receptors. Latter being supported by groups showing an effect of oestradiol on human epidermal keratinocytes, with increasing phosphorylisation levels of ERK1 and ERK2 kinases within 15

minutes.[157] It will be interesting to investigate this further and determine if vaginal skin also not only reacts via ERα and ERβ receptors but also through membrane bound equivalents. In the present study it was hypothesised that the mRNA for ERα would be upregulated after oestrogen treatment. Although elastin and MMP2 mRNA were increased ERα was not. However, we could show that the vaginal skin was producing mRNA for ERα permitting responsiveness to oestrogen either through intracellular and genomic action or through transmembrane receptors.

It has been found that hormone replacement therapy (HRT) increases epidermal thickness in female human epidermal skin after six months of oral oestrogen intake[158] and also increases hydration of skin whether being applied orally or topically.[159] ,[160] Furthermore, in a randomized, double blind, placebo controlled trial carried out by Sauerbronn et al in 2000, an increase of 6.49% of collagen content in epidermal human skin was demonstrated after six months of treatment with oral oestrogens.[161]. A study by Brincat et al undertaken in 1987 confirms the findings. It further shows that in women with initial low collagen content, oestrogen is initially therapeutic at first but later becomes solely prophylactic whilst in women with only mild collagen loss it is only prophylactic. The optimum collagen content was seen after two years of oestrogen treatment in this study which examined a time period of two to ten years of treatment.[162] The same effects were demonstrated in topical application of oestrogen in 1995 by Varila et al. Further, an increase in collagen synthesis was demonstrated with raised collagen I and III levels. The same group also was able to show an increase in tropoelastin and fibrillin mRNA in skin of males and females and a decrease in MMP1 expression. The most important difference to systemically applied oestrogen is that topical oestrogen appears to only be effective on the area it is applied to. [163] [164]

Tissues of the pelvic floor act rather similarly when it comes to oestrogen response even though the histology is somewhat different. Keane et al showed that vaginal skin and endopelvic fascia have similar properties[136] and so vaginal wall tissue was examined assuming similar changes to the endopelvic fascia. Whether all of these effects found in epidermal skin could also apply for vaginal skin in prolapsed vaginal tissue is therefore of interest. Changes through oestrogen application as described so far would not only help with current symptoms but may slow down the destructive change in the prolapsed tissue and maybe in future studies could be used to prevent prolapse altogether. Medication that was easy to apply was used in present studies. Creams and oestrogen releasing vaginal rings are known to be difficult to use in vaginal prolapse. The application is more difficult as rings tend to fall out and creams are not reliable as the amount used cannot be measured reliably and varies significantly, even when administered with an applicator. The use of a small pessary that can be inserted with an applicator into the posterior vaginal fornix was easiest. As each pessary contains the amount of 25µg 17β-oestradiol the same amount of medication is used by each woman. Furthermore, 17β-oestradiol is an oestrogen that the body is familiar with and has a proven effect in atrophic vaginitis and thus the best option for use in this study.

A very recent study from 2009 by Soederberg[165] et al examined expression of small leucine-rich repeat proteoglycans and fibulin-5 in para-vaginal tissue of women with pelvic organ prolapse. The group found a 16 fold decrease in decorin mRNA expression (P=0.0001) and an 8 fold decrease in lumican expression (P=0.001) in pre-menopausal women. All women with POP showed lower gene expression of fibromodulin and fibulin-5, the latter being an elastin associated protein. Thus a substantially decreased expression for regulators of

collagen fibre assembly and impairment in elastic fibre assembly was detected through down regulation of fibulin-5. The women were partly pre-and post menopausal leaving the question open whether hormonal profiles masked the results. Other studies, conducted in 2006 by Goepel et al[166], showed that paravaginal tissue from stress incontinent women showed fragmented distribution of elastin. Further studies could examine more closely which elastin fragments are affected by oestrogen to establish more focused support.

As oestrogen appears to be effective and improve skin of menopausal women (see above) and as shown in our study, ECM constituents of vaginal skin as well, the question is now could the type of oestrogen be more appropriately tailored to enhance the beneficial effects. A more tailored oestrogen derivative perhaps belonging to the group of Selective Estrogen Receptor Modulators (SERMs) could now be studied or perhaps even a cocktail of SERMs that promote different characteristics. Maybe, for instance,one would lower the collagen XVII for remodelling while another increased collagen XVII and adhesiveness after the reshaping and remodelling had taken place. It is known that SERMs can modulate oestrogen receptors in a tissue specific manner and are acting differently on varying tissues. Tamoxifen, a non-steroidal triphenylethylene is widely used in the management of hormonally responsive breast cancer. There it acts as an antagonist of oestrogen whereas in endometrial tissue it is a strong agonist and thus increases the risk of endometrial cancer by three fold [167]. Overall SERMs act as agonists for tissues such as bone, liver and the cardiovascular system, while their antagonistic effects are shown in brain and breast tissue. Yet, they have mixed effects on uterine tissue. Raloxifene, a benzothiopene, lacks uterotrophic activity and can thus be used as prevention of osteoporosis without these side effects.

The differences are found in the action of the SERMs. Tamoxifen for example activates the AP 1 target gene in uterine cells whereas raloxifene does not have this ability. SERMs act on skin. It is known that the expression of ERα and ERβ expression are modulated by tamoxifen and raloxifene[168] It is important to understand how SERMs also act on different tissue types to help create new SERMs with more favourable profiles, selected efficacy and side effect characteristics.

In conclusion, we were able to show that black women have preferential expression of elastic components that are likely to contribute to the lower prevalence of stress incontinence and pelvic organ prolapse compared with white women. In white women with vaginal prolapse an increase in remodelling and expression of elastin was found with the support of oestrogen in order to try and promote some of the beneficial characteristics of a black woman's vaginal skin. Perhaps these beneficial effects could be enhanced further with alternative oestrogen type compounds or by adding growth factors or cytokines that can also promote ECM synthesis. Further enhancement could occur with the use of matrix type scaffolds that are presently being investigated with different tissues (stem cell seeding is also being investigated with such scaffolds). A combination of such techniques could well be the future for alleviating the suffering associated with pelvic organ dysfunction. The present studies help to identify optimum characteristics to resist stress incontinence and pelvic organ prolapse and show the ability of oestrogen type compounds to produce some of the beneficial characteristics.

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Appendices

1. Kings Health Questionnaire for Pelvic Organ Prolapse (POP)
2. Calculation Matrix for Questionnaire
3. Symptom Questionnaire
4. Preliminary outcome of QoL assessment for the Vagifem study

THE KING'S HEALTH QUESTIONNAIRE PROLAPSE

1. How would you describe your health at the present?

Please tick one answer

Very good	⬭
Good	⬭
Fair	⬭
Poor	⬭
Very poor	⬭

2. How much do you think your prolapse problem affect your life?

Please tick one answer

Not at all	⬭
A little	⬭
Moderately	⬭
A lot	⬭

Please turn the page

2. Below are some daily activities that can be affected by prolapse problems. How much does your prolapse problem affect you? We would like you to answer every question. Simply tick the box that applies to you

3. ROLE LIMITATIONS	**1 Not at all**	**2 Slightly**	**3 Moderately**	**4 A lot**
A. Does your prolapse problem affect your household tasks? (e.g. cleaning, shopping etc.)	⬭	⬭	⬭	⬭
B. Does your prolapse problem affect your job, or your normal daily activities outside the home?	⬭	⬭	⬭	⬭

4. PHYSICAL/SOCIAL LIMITATION	**1 Not at all**	**2 Slightly**	**3 Moderately**	**4 A lot**
A Does your prolapse problem affect your physical activities (e.g. going for a walk, running, sport, gym etc)?	⬭	⬭	⬭	⬭
B. Does your prolapse problem affect your ability to travel?	⬭	⬭	⬭	⬭
C. Does your prolapse problem limit your social life?	⬭	⬭	⬭	⬭
D. Does your prolapse problem limit your ability to see and visit friends?	⬭	⬭	⬭	⬭

5. PERSONAL RELATIONSHIPS	**0** Not Applicable	**1** Not at all	**2** Slightly	**3** Moderately	**4** A lot
A. Does your prolapse problem affect your relationship with your partner?	⬭	⬭	⬭	⬭	⬭
B. Does your prolapse problem affect your sex life?	⬭	⬭	⬭	⬭	⬭
C. Does your prolapse problem affect your family life?	⬭	⬭	⬭	⬭	⬭

6. EMOTIONS	**1** Not at all	**2** Slightly	**3** Moderately	**4** Very much
A. Does your prolapse problem make you feel depressed?	⬭	⬭	⬭	⬭
B. Does your prolapse problem make you feel anxious or nervous?	⬭	⬭	⬭	⬭
C. Does your prolapse problem make you feel bad about yourself?	⬭	⬭	⬭	⬭

7.SLEEP/ENERGY	**1** Never	**2** Sometimes	**3** Often	**4** All the time
A. Does your prolapse problem affect your sleep?	⬭	⬭	⬭	⬭
B. Does your prolapse problem make you feel worn out and tired ?	⬭	⬭	⬭	⬭

8.Do you do any of the following to help your prolapse problem? If so how much?

	1 Never	2 Sometimes	3 Often	4 All the time
A. Use tampons/pads/ firm knickers	⬭	⬭	⬭	⬭
B. Do you push up the prolapse?	⬭	⬭	⬭	⬭
C. Do you have pain due to the prolapse?	⬭	⬭	⬭	⬭
D. Does the prolapse prevent you from standing?	⬭	⬭	⬭	⬭

3. We would like to know what your prolapse problems are and how much they affect you. From the list below choose only those problems that you have at present. Leave out those that don't apply to you.

How much do they affect you?

FREQUENCY: going to the toilet very often

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

NOCTURIA: getting up at night to pass urine

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

URGENCY: a strong and difficult to control desire to pass urine

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

URGE INCONTINENCE: urinary leakage associated with a strong desire to pass urine

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

STRESS INCONTINENCE: urinary leakage with physical activity eg. coughing, running

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Feeling a bulge/lump from or in the vagina

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Heaviness or dragging feeling as the day goes on from the vagina or the lower abdomen

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Vaginal bulge interfering with you emptying your bowels

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Discomfort in the vagina which is worse when standing and relieved by lying down

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Poor urinary stream

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Straining to empty your bladder

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Urine dribbles after emptying after emptying your bladder

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

3a. Please write down if you have any of the following symptoms and mark how much these affect you.

Bowel not feeling completely empty

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Constipation, difficulty in emptying your bowels

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Straining to open your bowels

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Vaginal bulge which gets I the way of sex

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Lower backache worsens with vaginal discomfort

1. Not applicable	2. A little	3. Moderately	4. A lot
⬭	⬭	⬭	⬭

Do you help emty your bowels with your fingers?

1. Not applicable	2. A little	3.Moderately	4. A lot
⬭	⬭	⬭	⬭

How often do you open your bowels?

1. More than Once/day	2. Once a day	3. Once every two days	4. Once/week or more
⬭	⬭	⬭	⬭

Thank you for your time!

To Calculate Scores

PART 1

1) General Health Perceptions

Very good	1
Good	2
Fair	3
Poor	4
Very poor	5

Score = ((Score to Q1 – 1)/4) x 100

2) Prolapse Impact

Not at all	1
A little	2
Moderately	3
A lot	4

Score =((Score to Q2 – 1)/3) x 100

PART 2

Individual scores as recorded at the top of each column of possible responses

3) *Role limitations*

Score =(((Scores to Q 3A + 3B) – 2)/6) x 100

4) *Physical limitations*

Score =(((Scores to Q 4A + 4B) – 2)/6) x 100

5) *Social limitations*

[If 5C >/= 1] Score =

(((Score to Q 4C + 4D + 5C) – 3)/9) x 100

[If 5C = 0] Score =

(((Score to Q 4C + 4D) – 2)/6) x 100

6) *Personal relationships*

[If 5A+5B >=2] Score =

(((Scores to Q 5A + 5B) – 2)/6) x 100

[If 5A+5B =1] Score =

(((Scores to Q 5A + 5B) – 1)/3) x 100

[If 5A+5B =0] Treat as missing value

7) *Emotions*

Score = (((Score to Q 6A + 6B + 6C) – 3)/9) X 100

8) *Sleep / energy*

Score = (((Scores to Q 7A + 7B) – 2)/6) x 100

9) *Severity measures*

Score = (((Scores to Q 8A + 8B + 8C + 8D) – 4)/12) x 100

PART 3

Scale	*score*
N/A	0
A little	1
Moderately	2
A lot	3

Symptom questionnaire pre and post surgery

Pre surgery

Obstetrics and Gynaecology Directorate
Urogynaecology
G Level, Mailpoint 105
Princess Anne Hospital
Coxford Road
Southampton SO16 5YA

Tel: 023 8079 8967
Fax: 023 8079 4801

A randomised trial examining the influence of vaginal oestrogen on symptoms, signs, connective tissue metabolism and surgical outcome in wo men with prolapse.

Pre-operative symptoms questionnaire

Visit 1 □ at time of surgery (3 months) □

Name: _____ DOB: _____

Profession: _____

Weight: _____

Hormonal status pre-operatively:

HRT: opposed □ unopposed □

Topical oestrogen: Vagifem □ other □ _____

Medical history:

Surgical history: Chronic cough: Y □N □

Obstetric history:

Parity	Birth weight	Mode delivery	Episiotomy/Tear	PFE

Symptoms pre-operatively:

Prolapse: Dragging sensation □

Pain/ discomfort □

Awareness of a lump □

Backache □

Urinary:

Urgency: mild □ moderate □ severe □

Urge incontinence: mild □ moderate □ severe □

Frequency: voids/day_____

Incomplete emptying: Y □ N □

Stress incontinence: Y □ N □

Nocturia: voids/night_____

Recurrent UTIs: Y □ N □

Voiding difficulties: straining □ poor stream □ incomplete emptying □

Bowel function: normal □ constipation □ loose □ recent change □

Mechanics: normal □ straining □ laxatives □

Digitation: none □ vaginally □ rectally □

Incontinence: none □ gas □ liquid□ solid □

Rectal symptoms: bleeding □

Soiling: Y □ N □

Difficulty wiping clean: Y □ N □

Sexual functions: not active □ no problems □ dyspareunia □

lack of sensation □ dryness □

Examination:

Aa	Ab	C
Gh	Pb	Tvl
Pa	Bp	D

Vulval skin: healthy □ red □ sore□ excoriated □

Vaginal mucosa: well oestrogenised □ poorly oestrogenised □ atrophic □

Perineum: healthy □ scarred □ atrophic □

Vaginal capacity: normal □ reduced □

Pelvic floor contraction: nil □ flicker □ moderate □ good □ strong □

Pelvic floor exercises pre op: Y □ N □

Post surgery

Obstetrics and Gynaecology Directorate
Urogynaecology
G Level, Mailpoint 105
Princess Anne Hospital
Coxford Road
Southampton SO16 5YA

Tel: 023 8079 8967
Fax: 023 8079 4801

A randomised trial examining the influence of vaginal oestrogen on symptoms, signs, connective tissue metabolism and surgical outcome in women with prolapse.

Post-operative symptoms questionnaire

Visit 2 (6/52 post op) □ Visit 3 (3 months post op) □ Visit 4 (6 months post op) □ Visit 5 (12 months post op) □

Name: _____ Dob: _____ Age:___

Profession: _____

Weight: _____ Operation date:_____

Surgeon: _____

Type of Surgery: anterior repair □ posterior repair □ ant.& post. repair □

Use of Mesh: anterior □ posterior □

Other procedure: _____

Prolapse recurrence: Y □ N □

Anaesthesia: GA □ Spinal □ LA □

Complications intra/post op:_____

Time to recover to normal activity:_____

Symptoms post-operatively:
Prolapse: Dragging sensation □
Pain/ discomfort □
Awareness of a lump □
Backache □

Urinary:

Urgency mild □ moderate □ severe □

Urge incontinence mild □ moderate □ severe □

Frequency voids/day_____

Stress incontinence Y □ N □

Nocturia: voids/night_____

Recurrent UTIs Y □ N □

Voiding difficulties straining □ poor stream □ incomplete emptying □

Bowel function: normal □ constipation □ loose □ recent change □

Mechanics: normal □ straining □ laxatives □

Digitation: none □ vaginally □ rectally □

Incontinence: none □ gas □ liquid□ solid □

Rectal symptoms: bleeding □

Soiling: Y □ N □

Difficulty wiping clean: Y □ N □

Sexual functions: not active □ no problems □ dyspareunia □

lack of sensation □ dryness □

Examination:

Aa	Ab	C
Gh	Pb	Tvl
Pa	Bp	D

Vulval skin: healthy □ red □ sore□ excoriated □

Vaginal mucosa: well oestrogenised □ poorly oestrogenised □ atrophic □

Perineum: healthy □ scarred □ atrophic □

Vaginal capacity: normal □ reduced □

Pelvic floor contraction: nil □ flicker □ moderate □ good □ strong □

Pelvic floor exercises post op: Y □ N □

QoL Summary:

Overall 49 women were recruited for the study to assess the symptoms and quality of life before and after Vagifem treatment.

The power calculation demonstrated that 105 women were needed:

If we use symptom reduction as an outcome measure and we postulate a 40% reduction in symptoms (we would expect more) with a power of 0.8 and a significance level of 0.05 we need 30 women in each arm.

The recurrence rates of prolapse are reported as high as 40% at one year. Using similar calculations we should have sufficient patients to detect a significant difference despite dropouts.

The final data would still need to be collected to establish statistical significance and report a change in symptoms as well as a reduction in recurrence rate of pelvic organ prolapse with Vagifem treatment. The study will be followed up to complete the data.

Overview over Methods from original trial protocol:

The patients for the study will be recruited from the waiting list for an anterior and or posterior repair in the Princess Anne Hospital. We will randomise 105 post-menopausal women. Patients with previous vaginal surgery, history of malignancy, collagen diseases and stress incontinence will be excluded. Patients will be asked to fill a QoL (quality of life) questionnaire and a standardised symptoms questionnaire. A vaginal examination to assess the stage of prolapse will be performed in accordance with the ICS standard (POP Q score). Then they will be randomised in two groups, the study group and the control group in a 2:1 ratio, respectively. The study group will receive a pelvic ultrasound scan to measure endometrial thickness and will be treated with topical vaginal oestrogens (25 mcg of

oestradiol pessaries twice weekly) and the control group will receive no treatment. A small punch biopsy (5mm x 5mm) from vaginal skin will be obtained under local anaesthetic from the patients in the study group. After three months of treatment surgery will be performed and a second biopsy will be obtained during the procedure from patients in the study group only and 4mls of blood will be obtained by venous puncture to assess the oestrogen blood level.

All the biopsies will be analysed for:

1. Collagen metabolism by measuring matrix metalloproteinases activity and their inhibitors.
2. The expression of the matrix remodelling cytokines within the tissue.
3. Immunocytochemistry staining for the ratio of collagen I:III.
4. Oestrogen receptor expression in the tissues.

The patients in the study will be randomised after the operation into two groups. One will continue with the oestrogen pessaries and the other one will discontinue the treatment. The investigator will still be blind to the second randomisation and will perform all the assessments.

Patients will be followed up at 1, 3, 6 and 12 months post surgery. A symptom questionnaire and a vaginal examination will be performed in each visit. A QoL questionnaire will be completed at 6 and 12 months visits. We can therefore look at the effect of oestrogen versus no treatment after 3 months treatment and the effect of oestrogen in preventing recurrent prolapse. Also repeated ultrasound scans will be performed for the patients in the study group at 6 months and 12 months post surgery to assess endometrial thickness.

Conclusions and Outlook:

As only 49 women were recruited and of these only 28 had filled in both required questionnaires, no statistically significant data could be obtained considering Quality of life (QoL). Data for the 16 women that had biopsies taken were taken and calculated (See table below).

Kings Health Questionnaire preliminary outcome:

King's Health questionnaire	pre Vagifem	post Vagifem
General health perceptions score	33.6	25.0
Impact on life score	60.2	55.5
Role limitations score	44.3	46.5
Physical limitations score	53.2	49.4
Social limitations score	46.4	43.6
Personal relationships score	46.3	44.6
Emotions score Sleep/energy score	37.9	34.4
Prolapse severity measures score	48.9	45.7
Urinary symptoms score	20.8	21.6
Frequency	1.7	1.5
Nocturia	0.2	0.3
Urgency	1.2	0.2
Urge incontinence	1.8	1.3
Stress incontinence	0.00	0.00
Bulge/Lump	2.8	2.4
Dragging	2.4	2.0
Emptying of bowels	2.3	2.7
Vaginal Discomfort	2.5	2.1
Straining	1.3	1.3
Urine dribbling	1.7	2.1
Incomplete bowel emptying	2.2	2.3
Constipation	2.6	2.1
Straining BO	1.4	1.5
Bulge in way of sex	2.3	2.1
backache	1.3	1.6
digitation	0.3	0.2
BO frequency	2.6	2.5

Table 1.1 Appendix, Mean for the QoL Assessment. The grey cells show an increase in the mean score and thus positive changes. Significance could not be established.

The preliminary trend, after comparing the current data for the first three months of follow up, is promising though. It appears that Vagifem indeed is improving symptoms and QoL prior to surgery in a majority of cases, even though there are not sufficient data to determine significance. There is no trend to suggest a decreased

recurrence rate as yet but certainly no deterioration of QoL was reported in the group using Vagifem for 18 months after surgery.
Overall Vagifem was well accepted by the patients and no significant side effects could be detected. Endometrial thickness (EMT) was measured to assess oestrogen influence. Two women had an increase in thickness and one required a hysteroscopy for postmenopausal bleeding. In the first two cases, an increase of EMT of 2mm was noted, but hysteroscopy was not required. In the latter case, bleeding started 2 weeks after Vegifem therapy, but an urgently organised hysteroscopy demonstrated normal intrauterine cavity findings. No tissue could be obtained for histology and the bleeding settled down quickly.

The continuation of the study would be extremely useful and interesting to not only demonstrate how vaginal oestrogens are changing the extracellular matrix of vaginal skin but also to examine if a regular use of this treatment should be established in clinical practice to support patients with their symptoms prior and after a prolapse surgery.