UNIVERSITY OF SOUTHAMPTON

COLLAGEN METABOLISM IN VAGINAL AND UTEROSACRAL TISSUE OF WOMEN WITH PELVIC ORGAN PROLAPSE

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ABSTRACT

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COLLAGEN METABOLISM IN VAGINAL AND UTEROSACRAL TISSUE OF WOMEN WITH PELVIC ORGAN PROLAPSE

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Pelvic organ prolapse is a debilitating condition affecting up to 15 per cent of the female population. Recent studies have suggested inherent abnormalities in collagen metabolism found in the vaginal skin may be causing the prolapse. The present study investigates the presence of markers of collagen metabolism in supporting structures of the uterus in addition to vaginal skin. The hypothesis is that changes in collagen metabolism in uterosacral ligaments is reflected in the vaginal skin.

Matrix metalloproteinases (MMP-2 & 9) content, concentrations of the inhibitor of MMP-2 (TIMP-2) and hydroxyproline were measured in biopsies of vaginal skin and uterosacral ligaments from women with prolapse (n=13) and matched controls (n=13). Oestrogen receptor expression within the smooth muscle of the uterosacral ligaments was also measured using immunohistochemical techniques and quantified.

There was a significant increase in pro MMP-2 expression in the vaginal skin of women with prolapse compared with controls ($p< 0.05$). There were small but not significant increases in the expression of active MMP-2, pro-MMP-9 and TIMP-2 in vaginal skin from women with prolapse. For uterosacral ligaments, both pro-MMP-2 and active MMP-2 expression were increased in samples from the women with prolapse. However, in contrast to samples of vaginal skin taken from the same women, this did not reach statistical significance. No significant difference in hydroxyproline content was found between control and prolapse. Interestingly, there were significant correlations between pro-MMP-2, active MMP-2, MMP-9 and TIMP-2 expression in vaginal skin with their expression in uterosacral ligaments ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.01$ respectively). Oestrogen receptors (alpha) were present within the nuclei of smooth muscle bundles in the uterosacral ligaments, but not detected in smooth muscle of blood vessels. There was no difference in oestrogen receptor expression in the uterosacral ligaments of women with prolapse compared with age matched controls.

In conclusion, evidence was shown for elevated matrix metalloproteinase in the vaginal skin of women with prolapse. Although similar trends occurred in uterosacral ligaments this did not reach statistical significance. Nevertheless, the fact that strong correlations existed between vaginal skin and uterosacral ligaments for markers of collagen metabolism suggested that changes occurring during the prolapse in uterosacral ligaments were reflected in vaginal skin. Alterations in collagen turnover are likely to be exaggerated in the vaginal tissue as it is less resistant to stretching. The expression of oestrogen receptor alpha did not appear to change in prolapse tissue but their presence in uterosacral ligaments suggest they may be a target for oestrogen.

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Abbreviations and key to diagrams

APES	aminopropyltriethoxysilane
α-SMA	alpha smooth muscle actin
ATFP	arcus tendineus fascia pelvis
AU	arbitrary unit
ABC	avidin-biotin complex
cm	centimetres
ct	connective tissue
Da	daltons
DAB	diaminobenzene
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EMG	electromyography
GAG	glycosaminoglycan
Gh	genital hiatus
GSI	genuine stress incontinence
H&E	haemotoxyllin and eosin
HCl	hydrochloric acid
HHL	histidino- hydroxylysinonorleucine
HLKNL	hydroxylysino-keto-norleucine
HLNL	hydroxylysinonorleucine
HRT	hormone replacement therapy
ICS	International Continence Society

Ig	immunoglobulin
IL	interleukin
Kda	killodaltons
LMP	last menstrual period
mg	milligrams
ml	millilitres
mmol	millimoles
MMP	matrix metalloproteinase
nm	nanometers
ng	nanograms
PBS	phosphate buffered saline
Pbl	perineal body length
PoPQ	Pelvic organ prolapse questionairre
PNTML	pudendal nerve terminal motor latency
RER	rough endoplasmic reticulum
SM	smooth muscle
TIMP	tissue inhibitor of metalloproteinase
TGF	transforming growth factor
TNF- α	tumour necrosis factor alpha
Tvl	total vaginal length
µg	micrograms
µl	microlitres
UTI	Urinary tract infection
Zn	zinc

Presentation of work related to thesis

Invited Lectures:

C. Phillips. The Role of the Extracellular Matrix in the Aetiology of Genitourinary Prolapse and Incontinence. *RUGS, Royal College of Obstetricians and Gynaecologists*, London, 1998.

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INTRODUCTION

Chapter 1:

Background

Contents to Chapter 1:

Chapter 1: Background

Section 1: Pelvic organ prolapse

Genitourinary prolapse is a common and distressing condition affecting up to 15 percent of the female population. It is defined as the protrusion of a pelvic organ or structure beyond its normal confines within the pelvis. Surgery is the mainstay of treatment for prolapse. Uterovaginal prolapse is responsible for around 20% of women on waiting lists for major gynaecological surgery (Cardozo et al, 1995). The morbidity, mortality and socio-economic problems associated with major surgery are of great concern when addressing the treatments of genitourinary prolapse. Research that improves our knowledge of the condition could help reduce the incidence of prolapse or improve therapeutic measures.

Olesen et al (1995), performed a retrospective cohort study of 395 women undergoing surgical treatment for prolapse and incontinence during 1995. They showed a woman has a 11.1% lifetime risk of undergoing a single operation for prolapse or incontinence by the age of 80. The major risk factors associated with the development of prolapse were increasing age and parity, whilst smoking and obesity were secondary factors. Twenty nine percent of women required re-operation for recurrent prolapse, indicating a high failure rate in primary surgical correction of prolapse.

Mant et al (1997), studied a cohort of 17,032 women who attended family planning clinics between 1968 and 1974, aged between 25 and 39 at study entry (Oxford FPA study). They showed the incidence of hospital admission with prolapse was 2.04 per 1000 person-years of risk. Significant risk factors associated with prolapse were age and parity as well as increased weight. Significant trends were seen for smoking and obesity, but not for social class, oral contraceptive use or height. Recurrence rates were again shown to be high, rising with time after hysterectomy. Cumulative risk of recurrence rose from 1% after three years to 5% after fifteen years. Women have a 5.5 times higher risk of recurrence if their hysterectomy is for prolapse rather than other pathology. It should be noted that there are a number of differences between the cohort of women studied and the general population. Women at entry were less likely to have chronic disease, to smoke heavily, to be grossly overweight, or to be in lower social classes than would be expected in the general population. These differences would tend to suggest that risk of prolapse may be higher in the general population than observed in the Oxford-FPA study. Another study from the Womens' Health Initiative of around twenty-seven thousand women showed the incidence of uterine prolapse was 14 percent , cystocele; 34 percent and rectocele; 18% of women (Hendrix et al, 2002). Prolapse is rare in Afro-Carribean women compared with a Caucasian population (Bump, 1993).

High failure rates, morbidity associated with major surgery as well as financial implications for the health service all suggest that current management of prolapse is less than satisfactory. Current opinion suggests that a better understanding of the disease processes involved in prolapse may help develop alternative, more effective treatments for

the condition. During the introduction of this thesis the structures involved in supporting the pelvic organs will be discussed, along with the multifactorial aetiology of pelvic organ prolapse. Further sections will outline the role of connective tissue in pelvic support as well as the significance of collagen production and breakdown in the aetiology of the disease process.

1.1 Classification of Pelvic Organ Prolapse

Pelvic organ prolapse is classified according to the structure or organ that is protruding into, or beyond the vagina. This is summarised in table 1.I and figure 1.1.

Table 1.I Classification of prolapse

Uterus:	uterine prolapse/procidentia (body of the uterus)
Bladder:	cystocele
Urethra:	urethrocele
Rectum:	rectocele
Omentum & small bowel	enterocele
Vagina:	vault prolapse (post hysterectomy)

The extent of the prolapse is then graded according to the degree of descent of the protruding structure into the vagina. Grade 1 prolapse indicates the organ has descended into the vagina but has not reached the hymenal ring, grade 2 is up to the hymenal ring and

grade 3 indicates the structure is protruding beyond the hymenal ring. Grade 3 prolapse of the uterus is also called "procidentia" (see figure 1.2). The grading system allows the clinician to estimate the severity of the prolapse, however, this classification has high inter-observer differences. The International Continence Society (ICS) has developed a more specific and reproducible classification system for pelvic organ prolapse which is further explained in Section 2.5.

Figure 1.1 Classification of prolapse: reproduced from Campbell S & Monga A; Gynaecology by Ten Teachers, Arnold Press, 2000, with permission.

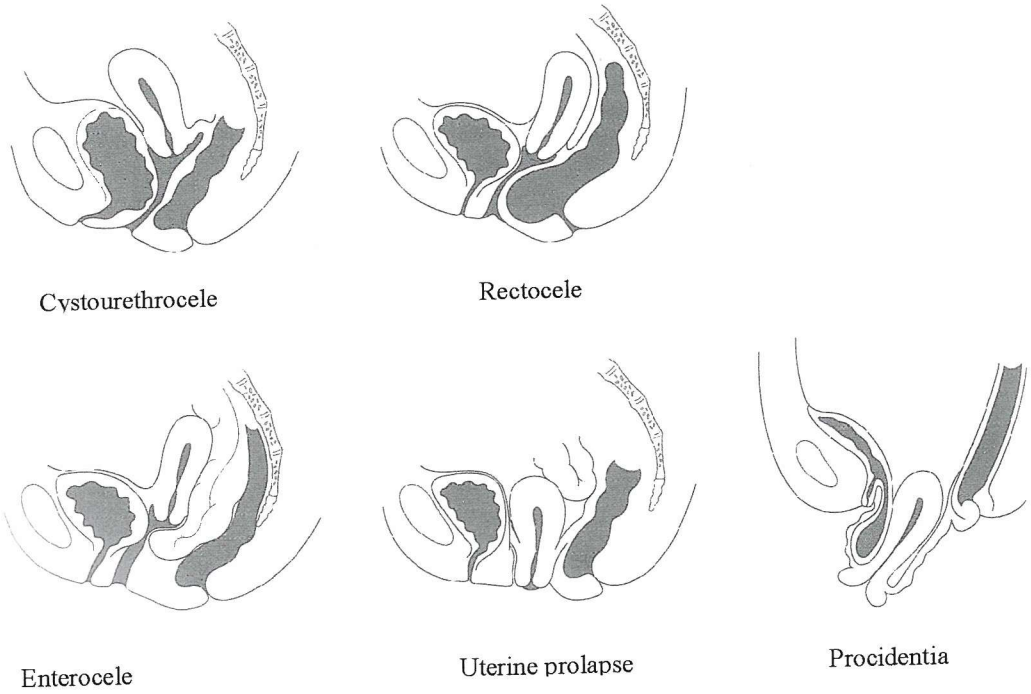
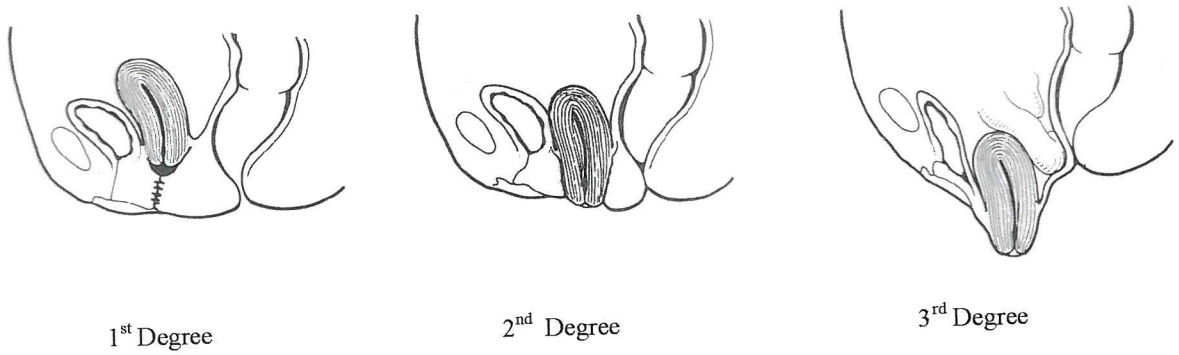


Figure 1.2 Grading of prolapse. Reproduced from Symonds M. Essential Obstetrics and Gynaecology, Churchill Livingstone, 1987, with permission.



1.2 Symptoms and management of pelvic organ prolapse

It is essential to obtain a detailed history by careful systematic enquiry. Often genitourinary prolapse is asymptomatic and only noted at a "well women" clinic. If this is the case little or no treatment is required. Common symptoms include:

General Symptoms

- Pressure
- Protrusion of tissue/dragging sensation (lump)
- Impaired coitus
- Difficulty in walking
- Back ache
- Pelvic pain
- Mucosal irritation/ulceration/bleeding/discharge

Symptoms specific to anterior wall prolapse

- Difficulty in voiding/incomplete bladder emptying/poor stream/UTI's
- Urinary incontinence (approximately 50% of women with cystocele have stress incontinence)
- Urinary frequency/urgency

Symptoms specific to posterior wall prolapse

- Difficulty in defaecating: incomplete bowel emptying, vaginal/perineal splinting, staining

Currently two instruments are under evaluation for measuring quality of life in relation to prolapse, but are not currently available in clinical practice (Hiller et al, 2002[1&2]). Questions regarding bowel and bladder function are imperative to elucidate concomitant stress urinary incontinence, voiding difficulties or bowel dysfunction. It is also important to ask whether the woman is sexually active as this has implications for surgery, as traditional surgical procedures for prolapse may reduce the woman's vaginal capacity. This is especially seen in the case of rectocele where plication of the levator ani performed during a traditional posterior colporrhaphy is associated with significant postoperative dyspareunia (Kahn et al, 1997).

After a detailed history has been obtained, the clinician would perform a thorough physical examination. This would include examination of the abdomen to exclude a pelvic mass, and vaginal and speculum examination to assess the severity of the prolapse. Vaginal examination is performed in the dorsal lithotomy position, whilst inspection using a Sim's speculum is carried out with the patient lying in the left lateral position. The examiner is looking to assess the perineal body, mobility of the urethrovesical junction, the presence and degree of urethrocele, cystocele, enterocele, rectocele as well as degree of descent of the uterus. Other factors that are important are signs of hypo-oestrogenism such as vaginal atrophy, loss of rugae and ulceration of the vaginal epithelium. Patients with poor perineal body and rectocele would have a rectal examination performed to corroborate vaginal findings. Bidigital examination is also useful to discriminate enterocele from rectocele.

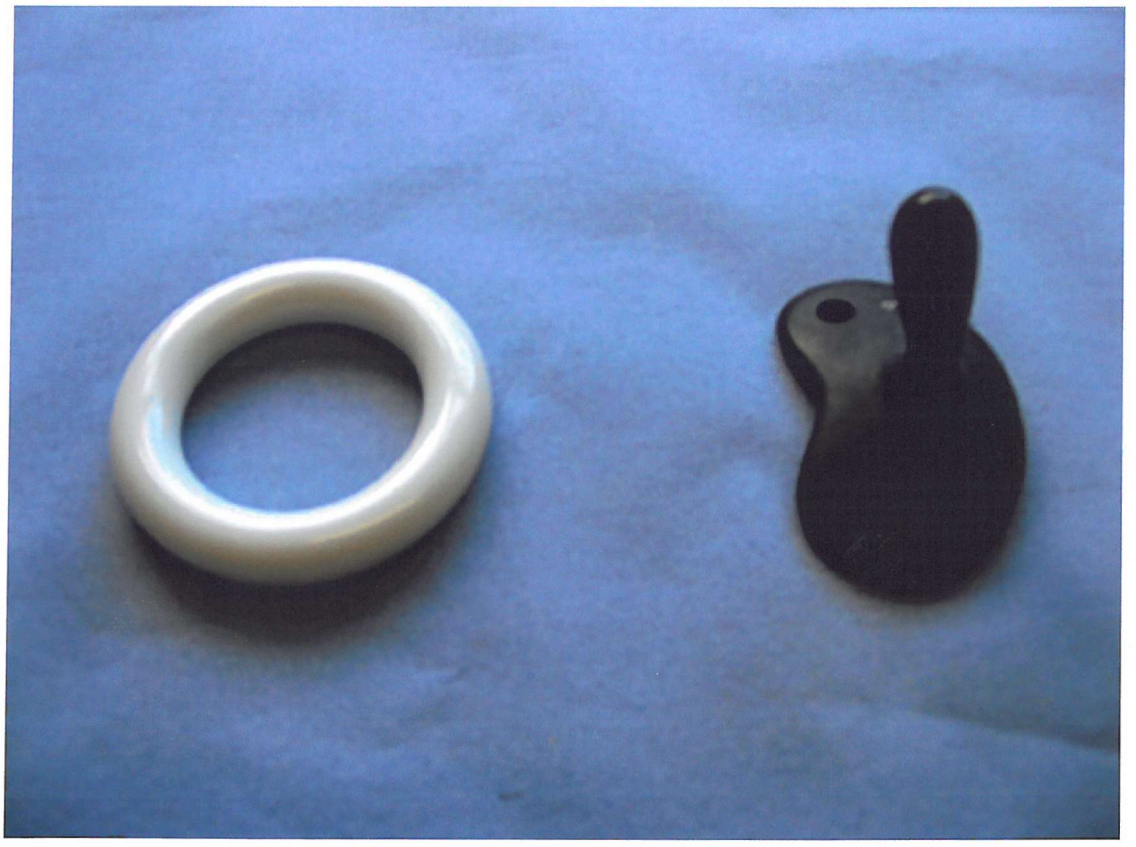
Prolapse is often associated with symptoms affecting the bladder, continence mechanisms and bowels. If a patient complains of concomitant urinary or bowel symptoms they may be referred for further investigation prior to embarking on surgery, especially if the clinician feels the symptoms are not attributed to the prolapse alone. Those with urinary symptoms often have urodynamic investigation as the presence of concomitant stress incontinence may require an additional or different surgical procedure to be performed. If the patient has symptoms of faecal incontinence; anorectal manometry, transanal ultrasound and electrophysiological studies may be arranged to help identify a defect in the anal sphincter. Defaecography and motility studies may be needed in women with motility problems as they may not perceive any improvement in bowel function after a rectocele repair.

Although the treatment of prolapse is mainly surgical, prevention and conservative means have been outlined below. Prevention of pelvic trauma at childbirth helps to prevent prolapse occurring later in life. Obesity, smoking or chronic cough all cause a state of raised intra-abdominal pressure which aggravates prolapse and so should be treated before embarking on treatment for prolapse. Pelvic floor exercises are useful in women complaining of mild genitourinary prolapse and concomitant stress incontinence, with success rates quoted from 50% to 60%, however their use in the treatment of established prolapse alone is ineffective.

Vaginal pessaries are particularly useful for the frail woman for whom surgery is not an option, however the frequency of pessary use is falling because of modern, safe general,

and regional anaesthesia, as well as the advent of colporrhaphy under local anaesthesia (Dorflinger et al, 2001). Pessaries can also provide symptomatic relief for those awaiting surgery, although intercourse may be difficult. Vaginal pessaries are most effective in women with good perineal support, which prevents the pessary being expelled spontaneously, or during the valsalva manoeuvre. Pessaries are made from an inert silicone or rubber and come in various shapes and sizes. The most commonly used is the ring pessary (see figure 1.3).

Figure 1.3 ***Ring and shelf pessaries***



Once conservative treatments have failed the mainstay of treatment is surgical. The aims of surgery are to restore anatomy, whilst preserving continence and maintaining coital function. Surgery should only be attempted once conservative means have either been tried, or at least discussed with the patient. When deciding on which procedure should be performed, the surgeon needs to assess the patient's medical and anaesthetic fitness as well as the need for concurrent surgery. The majority of repairs can be performed vaginally, but greater success rates can sometimes be achieved if the procedure is performed abdominally, however a laparotomy is associated with greater morbidity. The various surgical procedures are outlined in table 1.II.

Table 1.II Surgery for prolapse

Abdominal Procedures:

Uterine prolapse:	Total abdominal hysterectomy
Cystocele:	Paravaginal repair
Vault prolapse:	Sacrocolpopexy

Vaginal Procedures:

Uterine prolapse:	Vaginal hysterectomy
Cystocele:	Anterior colporrhaphy
Rectocele:	Posterior colporrhaphy
Enterocele:	Enterocele repair
Vault prolapse:	Sacrospinous fixation / iliococcygeus fixation

Current surgical techniques usually involve the plication of existing tissue with either absorbable or permanent sutures or the insertion of a synthetic or organic mesh. However, all sutures / meshes are then anchored to the patient's fascia, muscle or occasionally periosteum. Therefore, the success of surgery is determined by the following:

1. The choice of operation
2. The surgeon's attention to surgical detail
3. The integrity of the patients own tissues

If the patient's prolapse is caused by a weakness in the patient's own fascia then anchoring sutures to structurally impaired tissue may result in recurrence. This study addresses the connective tissue of the ligaments supporting the uterus in women with prolapse and compares this with the connective tissue in women with no prolapse.

1.3 Normal anatomy of pelvic support

Victor Bonney (1934) described three mechanisms of support that exist in the female pelvis: constriction, suspension and flap-valve closure.

1. Constriction of the vagina by the levator ani occludes the opening through which prolapse of pelvic structures occurs.
2. The angulation of the vagina, which allows it to close against the levator plate when the intra-abdominal pressure increases.
3. Fascia and ligaments suspend the uterus, bladder and bladder neck, and rectum to the pelvic sidewalls.

Muscular support.

The muscular support of the pelvic viscera is mainly provided by the levator ani muscles: pubococcygeus, iliococcygeus and ischiococcygeus. The iliococcygeus muscles form a sheet-like structure extending from the arcus tendineus anteriorly, travelling behind the rectum to meet a midline raphe fused posteriorly to the coccyx. The two muscles act as a diaphragm lying over the urogenital hiatus, upon which the pelvic viscera lie. The pubococcygeus component forms a sling-like structure around the urogenital hiatus and is involved in sphincteric mechanisms of the urethra. The control of muscle tone at rest and at stress is maintained by an intact nerve supply. The levator ani muscles are innervated by the second, third and fourth sacral nerve roots (S2,3,4). The pubococcygeus muscle, along with the external anal sphincter is innervated by branches of the pudendal nerve derived from the second, third and fourth sacral nerve roots (S2,3,4). When the levator ani muscles relax the urogenital hiatus widens and the fascia and ligaments are left holding the uterus and vagina in place. This has been confirmed using three-dimensional ultrasound imaging of the levator ani hiatus. Measurements taken of the levator hiatus, show that the hiatus is larger in women with prolapse than in those without prolapse' (Athanasiou et al, 1995).

Connective tissue support.

Integrity of the endopelvic fascia is also important in supporting the uterus, bladder and bowel. Anteriorly the pelvic fascia forms the pubourethral ligaments. These lie either side of the midline to form an aponeurotic plate supporting the cranial aspect of the proximal urethra. The pubourethral ligaments extend anteriorly becoming continuous with the

suspensory ligament of the clitoris. The endopelvic fascia then extends as a fibrous band running from the symphysis pubis anteriorly, widening posteriorly to attach to the ischial spine. This structure called the arcus tendineus fascia pelvis (ATFP), also known as the "white line", can easily be seen in the space of Retzius at colposuspension. The fascia extends continuously from the symphysis pubis anteriorly, the arcus tendineus and levator ani muscles laterally to the ischial spines posteriorly, enveloping the pelvic organs (See figure 1.5).

In certain areas the fascia condenses to form dense connective tissue called the uterosacral ligaments and the cardinal complex. The uterosacral ligaments provide support for the uterus posteriorly, the cardinal ligaments provide support laterally and the pubocervical ligaments provide support anteriorly (see figure 1.4). Condensations of fascia around the uterus are called the parametrium and around the upper vagina; the paracolpium. The endopelvic fascia is made up mainly of connective tissue and smooth muscle in various proportions according to site and structure. Campbell (1950), noted that the proportion of connective tissue to smooth muscle within the uterosacral ligaments increases nearer the sacrum. Range and Woodburne (1964), showed the cardinal ligaments contain perivascular connective tissue whereas the uterosacral ligaments are predominantly smooth muscle and connective tissue. Mengert (1936), demonstrated in experiments on cadavers that the parametrium, paracolpium and in particular the uterosacral ligaments are the most important areas of support for the uterus and vagina. DeLancey (1993) expanded on this to describe three levels of support of the pelvic viscera:

- Level 1 extends upper 1/3 of the vagina and the cervix suspending the uterus and vaginal apex to the lateral pelvic side walls
- Level 2 attaches the mid-portion of the vagina directly to the pelvic walls forming the pubocervical and rectovaginal fascia. The pubocervical fascia between the anterior vaginal wall and the bladder retains continuity with the arcus tendineus and the levator ani to form a "hammock" below the bladder neck and urethra (DeLancey 1990). This continuity is thought to be important in maintaining continence of urine as well as supporting the bladder neck and urethra (see figure 1.5).
- Level 3 direct attachment of lower portion of vagina to surrounding structures without paracolpium.

Loss of support at each level gives rise to different types of prolapse. Damage to the upper suspensory fibres of the paracolpium can lead to prolapse of the uterus and vaginal apex. Loss of level 2 support anteriorly would give rise to urethrocele, cystourethrocele or cystocele, often accompanied by stress incontinence. Loss of continuity of the rectovaginal fascia and the pelvic sidewall would lead to rectocele.

The aetiology of uterovaginal prolapse is probably multifactorial. Studies suggest that some women may be susceptible to developing prolapse after trigger events such as childbirth or with age. Support of the pelvic viscera is achieved by the interaction between the levator ani and the pelvic fascia. An analogy of a boat in dry dock has been used to explain the interaction between the levator ani muscles and the endopelvic fasia.

The water supports the weight of the boat whilst the mooring ropes hold it in position. The water is analogous to the levator ani muscles and the mooring ropes to the endopelvic fascia and suspensory ligaments. If the water level was to fall significantly then excessive forces would be placed on the mooring ropes. After childbirth neuromuscular damage causes a reduction in levator resting tone and a widening of the urogenital hiatus. The uterus and vagina therefore become increasingly supported by the endopelvic fascia of the parametrium and paracolpium. If the fascia is inherently weak or already damaged then the ligaments will stretch and prolapse will develop.

Figure 1.4 ***The uterosacral, cardinal and pubocervical ligaments which all provid support to the uterus. Reproduced from Gynaecology by ten teachers 15th Edn, Eds. Lewis TLT, Chamberlain GVP.1990 Arnold Publishers, with permission***

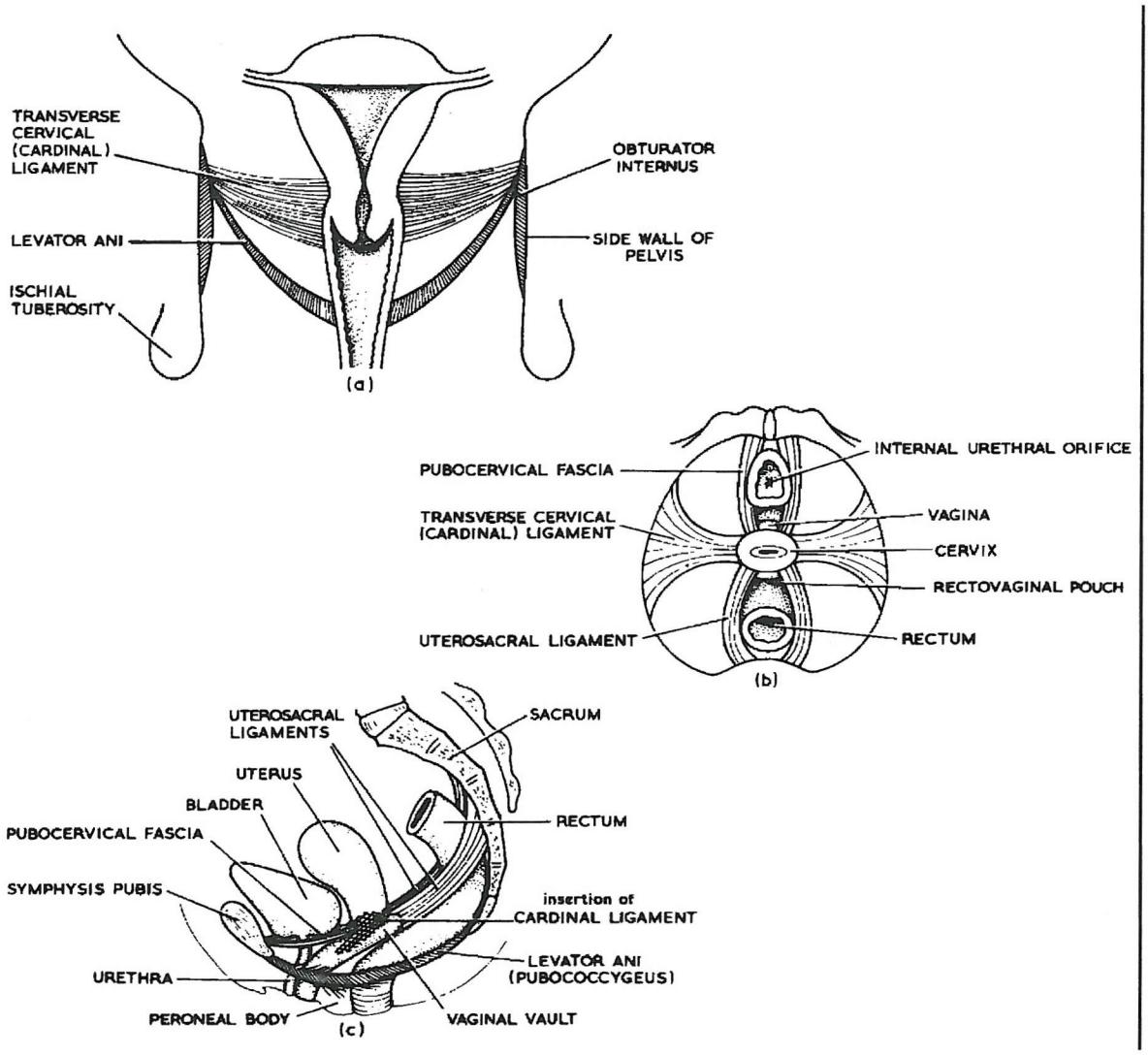
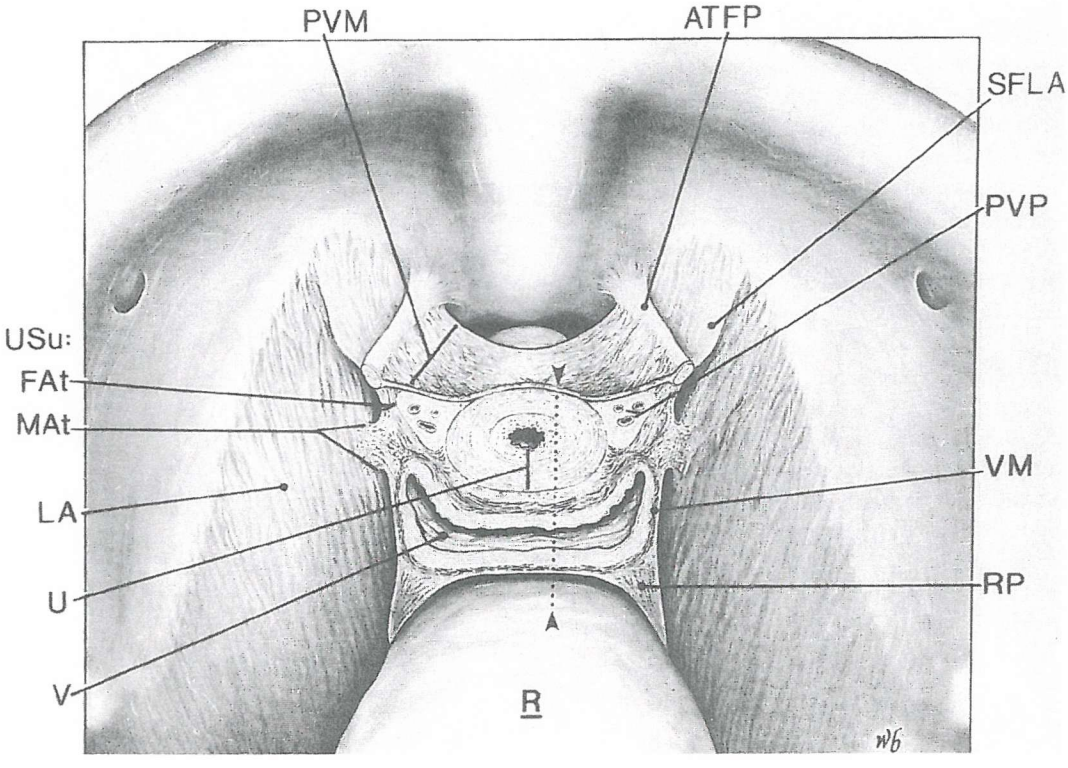


Figure 1.5 ***Cross section of the urethra (U), vagina (V), arcus tendineus fascia pelvis (ATFP), and superior fascia of levator ani (SFLA) just below the vessical neck.*** *Reproduced from De Lancey JOL, Neurourol Urodynam. 1989; 53-61, with permission*

The pubovesical muscles (PVM), paraurethral vascular plexus (PVP),,(USU): urethral supports, (PVP): pubourethral ligament, (Mat, Fat): muscular attachments,fascial attachments of levator ani, (R) rectum, (RP): rectal pillar, (VM): vaginal wall muscularis, (LA): levator ani).



1.4 Pathophysiology of prolapse

The aetiology of pelvic organ prolapse remains unclear, however the following factors have been implicated:

1. Mechanical trauma to the pelvic floor musculature
2. Damage to the nerve supply to the pelvic floor muscles
3. Insult to the endopelvic fascia

- Myogenic injury in pelvic organ prolapse

There is evidence that mechanical trauma causes rupture of the pelvic musculature at delivery. Recent studies using magnetic resonance imaging of the pelvic floor after vaginal delivery have demonstrated areas of muscle deficiency within the striated muscle of the pelvic floor as a direct consequence of childbirth (Debus-Tiede et al, 1993). Biopsies of levator ani from mice have shown characteristics of myogenic injury occurring after parturition (Yiou et al, 2001). Histological examination of cadaveric dissections taken from the pelvic floor show childbirth causes direct myogenic changes in the levator ani muscles (Dimpfl et al, 1998).

- Denervation injury in pelvic organ prolapse

Damage to the nerve supply of the pelvic floor caused by childbirth may cause progressive denervation of the musculature. Subsequent re-innervation of the pelvic floor leads to an altered function, morphology and neurophysiology. Histological, histochemical and electromyography studies have measured the degree of re-innervation and have assumed

this is the result of denervation caused by delivery (Gilpin et al, 1989; Smith et al, 1989). The exact nature of denervation injury is unknown but one mechanism may be pudendal nerve compression. The pudendal nerve innervates the levator ani muscles from the second, third and fourth sacral nerve roots (S2,3,4). During its course from S2,3,4 to the levators, the pudendal nerve takes a "U"-bend around the ischial spines which is a potential site for compression by the fetal head during childbirth. One can assess the degree of nerve injury by measuring the time taken for a motor response to an electrical evoked potential. Prolonged pudendal nerve terminal motor latency (PNTML) has been reported after childbirth, but may also be as a result of other pathologies, such as demyelination. Snooks et al (1984), showed that women having a vaginal delivery had significantly prolonged PNTML and greater perineal displacement on straining compared with controls. PNTML are further significantly prolonged in primiparas who had a forceps delivery rather than a normal vaginal delivery. Caesarean section had no effect on neurophysiology compared with controls (Snooks et al, 1984). However, although pelvic floor neuropathy has been demonstrated in many women immediately after delivery, most will recover neuromuscular function and only few will suffer long term problems. In 80% of women with initial prolonged PNTML, there is a reversal to normal by 2 months post-partum (Snooks et al, 1984). Snooks (1986) then repeated anal manometry, EMG and PNTML on 58% of these women five years later and showed a progressive increase in neuropathy with age. Sultan et al (1994), measured PNTML at 34 weeks gestation and at 6 weeks and 6 months post-partum. Their results supported the findings of Snooks et al, but also showed that PNTML can be prolonged in women who have a caesarean section after the onset of labour indicating that pudendal nerve damage may not be due to

pregnancy but labour, irrespective of vaginal delivery. In view of the conflicting data on PNTML, the reliability of PNTML measurement is already being challenged.

It is not known why neuromuscular function is regained in some women after childbirth while others continue to have permanent damage. Decreased neuromuscular function can be seen in women with greater perineal body descent on straining. It has been postulated that repeated stretching of the pudendal nerve by straining causes recurrent injury to the nerve, preventing it from recovery (Snooks et al, 1990, Sultan et al, 1994). However, although neuromuscular function decreases with age and parity, there appears to be no correlation between the degree of perineal descent and increase in PNTML (Ryhammer et al, 1998). Factors associated with greater pelvic floor nerve damage are parity, forceps delivery, prolonged second stage of labour, third degree perineal tears and macrosomia (Snooks et al, 1986).

Prolapse is often demonstrable in women with no evidence of direct myogenic injury or denervation injury. Indeed two per cent of women with genitourinary prolapse are nulliparous. This suggests that muscular and neurological injury cannot fully explain the pathophysiology of genitourinary prolapse. As already explained the muscles and ligaments work in conjunction to support the pelvic organs and there is now growing interest in investigating the nature of the endopelvic fascia and how it changes in women with pelvic organ prolapse.

Prior to discussing the literature regarding the role of connective tissue abnormalities in the aetiology of pelvic organ prolapse, the biochemistry of the extracellular matrix will be explained to put the range of abnormalities into perspective.

Section 2: The structure and function of connective tissue

The extracellular matrix (ECM) has two main constituents needed for functionality, namely the insoluble fibres which resist tensile forces and interfibrillar polymers which provide resistance to compressive forces, whilst allowing diffusion of small molecules in and out of the tissue. Collagen and proteoglycans are the main constituents of the extracellular matrix but there are other glycoproteins present, which are involved with organisation and interaction of the extracellular matrix with other cells.

1.5 Collagen: structure and assembly

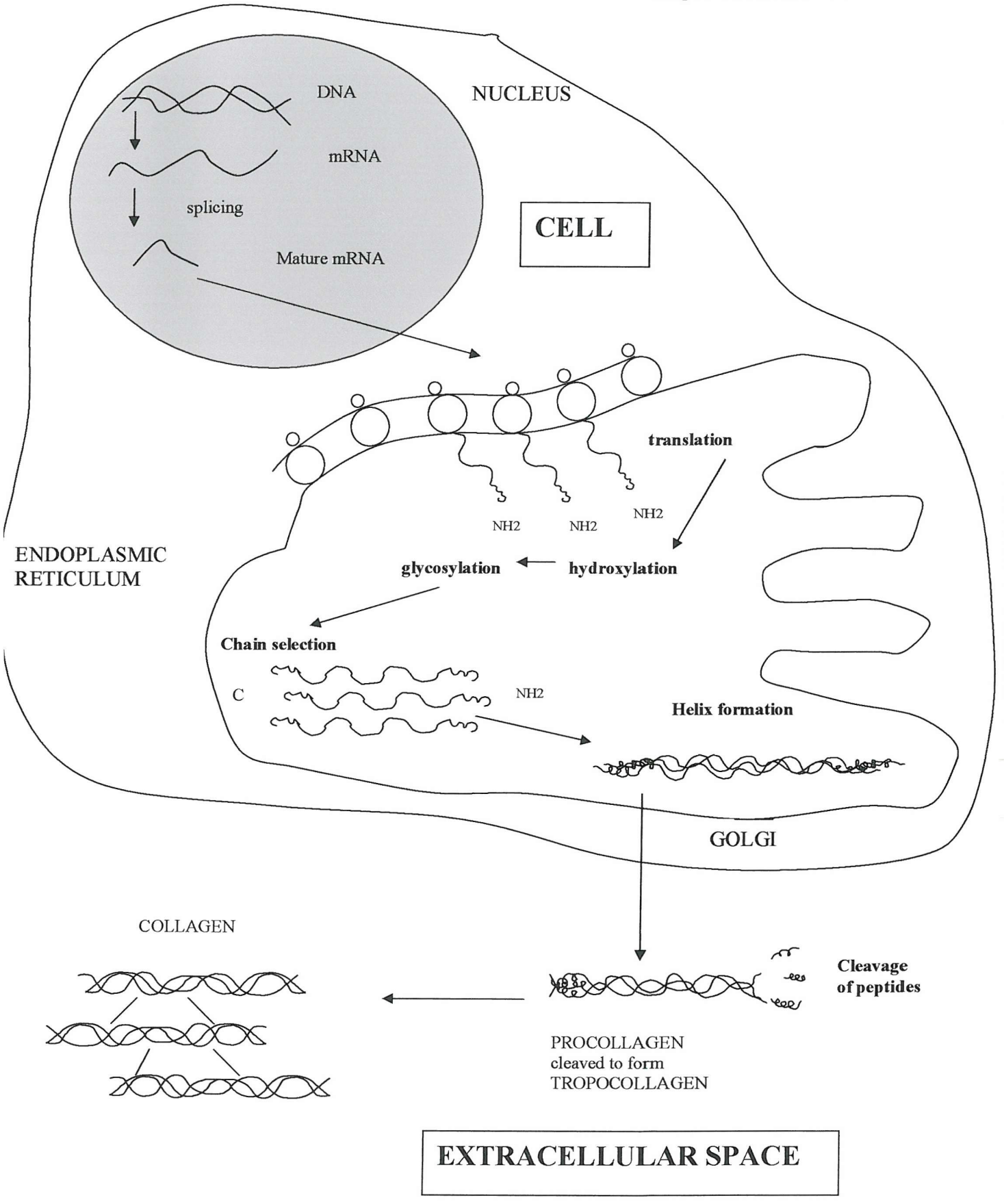
Collagen is the most abundant protein in the body, accounting for 30% of the body's total protein content. Derived from the Greek word meaning "glue", collagen is a fibrous protein high in tensile strength. To understand how collagen provides such strength to tissues, the structure and assembly of collagen needs to be examined

The protein collagen forms a characteristic tight triple-helical shape, which is consistent throughout all species in the animal world (Kielty et al, 1993). This is achieved by the insertion of the amino acid *glycine* at every third position in the amino acid chain (- X – Y – Gly -). Because glycine is such a small molecule, it allows the chain to form a tight helix. The specificity of this pattern means that mistakes or substitutions in the glycine insertion, will affect the structure and function of the fibres and ultimately the survival of the animal itself. The tight helix therefore is of primary importance to the function of

collagen. The two other amino acids that appear regularly in its structure are *proline* and *hydroxyproline*. These two amino acids provide additional strength and stability to the helix by forming cross-links between the collagen chains.

Collagen is initially assembled as a pro-protein inside the fibroblast cell and is then modified after production (see figure 1.6). Synthesis is initiated by transcription from specific genes coding for the polypeptide chains, followed by processing of the mRNA precursor and translation (synthesis of protein) of the α chains on the ribosomes. The α chains subsequently emerge from the ribosomes into the cisternae of the rough endoplasmic reticulum (RER), where they undergo a series of biochemical modifications of the chains. This modification is activated by a number of specific enzymes. One of the main modifications is the hydroxylation of *proline* in the α position to *hydroxyproline*, which provides collagen with its high content of hydroxyproline (about 14%). This hydroxylation is dependent on the availability of ascorbic acid (vitamin C) and deficiency in this can cause defective collagen: scurvy. As the hydroxyproline content of collagen remains constant through tissues the amount of hydroxyproline can be used to estimate the total collagen content. Whilst in the cisternae of the RER, disulphide bonds exist between the three α chains of pro-collagen. In this state the pro-collagen is still soluble and contains an extra length of polypeptide and C terminal at the ends of the chain. It is then transported through the golgi apparatus and larger vacuoles to the cell surface. During excretion from the cell pro-collagen peptidases remove the terminal peptide chains to form *tropocollagen* (see figure 1.6).

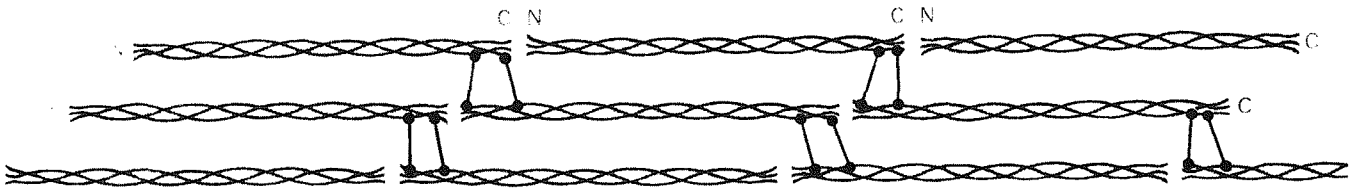
Figure 1.6 ***Steps in the synthesis of collagen.***



Tropocollagen is the basic building block of collagen consisting of three tightly woven strands. The tropocollagen rapidly aligns with other tropocollagens to form fibrils. These true fibrils give strength to the connective tissue. The tropocollagen units are laid end to end so the fibril "rope" could continue indefinitely. Another critical extracellular modification is the oxidation of lysine. This results in the formation of cross-links between the α chains of adjacent molecules and is the basis for the structural integrity of collagen. Adjacent fibril lengths are staggered so that no one region of the "rope" is weaker than another (see figure 1.7).

The cross-links are initially the divalent cross-links dehydro-hydroxylysinonorleucine (Δ-HLNL) and hydroxylysino-keto-norleucine (HLKNL). As the tissue matures these are converted to the more stable trivalent cross-links histidino-hydroxylysinonorleucine (HHL) and hydroxylysylpyridnoline (pyrid) respectively (Sell et al, 1989). The relative proportion of divalent to the mature trivalent cross-links can be measured to provide an assessment of the maturity of the tissue (Sell et al, 1989). Many fibrils are bundled together to form large collagen fibres. The number of chains and type of collagen is determined according to the tissue properties required.

Figure1.7 Location of cross-links between adjacent tropocollagen molecules.



1.6 Collagen types

It was once thought that all collagen was alike. However, with the advent of amino acid and cDNA sequencing techniques, over twenty types or classes of collagen have been identified (See table 1.III). The collagen class is determined by the composition of α-chains. For example, type I collagen is the most common, consisting of two alpha-1 (type 1) chains disulphide bonded to on alpha-2 (type I) chain.

The main types of collagen found in connective tissue are *fibrillar collagens, fibril associated collagens* and the *network forming collagens*. The *fibrillar collagens* include types I, II, III, V and XI. After being secreted into the extracellular matrix the collagen molecules assemble into ordered polymers called collagen fibrils, which are thin (10-300nm in diameter) structures, which can be many hundreds of micrometres long in mature tissues. The collagen fibrils then aggregate into larger cable-like bundles called collagen fibres which can be seen with a light microscope. Types IX and XII are called *fibril-associated collagens* as they decorate the surface of the collagen fibrils and are

thought to link the fibrils together and to other components of the ECM. Types IV and VII are *network forming collagens*: type IV assembles into a sheet-like structure and form a major constituent of the basal lamina. Type VII molecules form dimers that assemble into specialised structures called *anchoring fibrils*, which help attach the basal lamina of multilayered epithelia to the underlying connective tissue.

Table 1.III Types of collagen

Type	Chains	Characteristics	Distribution
I	α1(I),α2(I)	Fibres bundled, with high tensile strength	Skin, bone, tendons
II	α1(II)	Thin fibrils: structuaral protein	Cartilage, vitreous humour
III	α1(III)	Thin fibrils, pliable	Blood vessels, uterus, skin
IV	α1(IV),α2(IV)	Amorphous	All basement membranes
V	α1(V,α2(V)),α3(V)	Amorphous fine fibrils	Interstitial tissues, vessels
VI	α1(VI), α2(VI)	Function unknown	
VII	α1(VII)	Function unknown	
VIII	α1(VIII)	Probably amorphous	Endothelium specific
IX	α1(IX), α2(IX), α3(IX)	Possible role in cartilage maturation	Cartilage
X	α1(X)	Possible role in cartilage maturation	Cartilage

The main fibrillar collagens found in connective tissue are collagen type I and collagen type III. Type I collagen is strong and usually organised into large fibres and is usually found in large quantities in tissues needing strength, such as ligaments, fascia and tendons. Type III collagen is a trimer of three alpha –1 (type III) chains. In tissues, type III collagen forms smaller, more randomly organised fibres compared with type I collagen and is seen predominately in tissues that require a degree of flexibility such as the skin, aorta, lungs, uterus, fascia and ligaments. Most tissues are composed of varying amounts of both type I and type III collagen. The proportion of each type is determined by the structural requirements of the tissue.

1.7 Degradation of collagen

The collagenases are a group of enzymes that are involved in the extracellular breakdown of collagen. Since their discovery, they have been shown to be part of a larger family of enzymes involved in degradation of the extracellular matrix, known as the matrix metalloproteinases (MMP's). These are a family of metal-binding enzymes which are secreted in a *pro* form which then require extracellular activation. The MMP family has three major subgroups, the *interstitial collagenases*, the *gelatinases* and the *stromelysins* (see table 1.IV). There is a high degree of similarity between the enzymes in each group and between the groups, as well as similarity in domains covering the active and latent sites (see fig 1.8). One of the active sites is the conserved Zn^{2+} binding site HEXGHXXGXXH, as well as other regions of homology which are important in converting latent pro-forms to their active enzymes (Murphy et al, 1993).

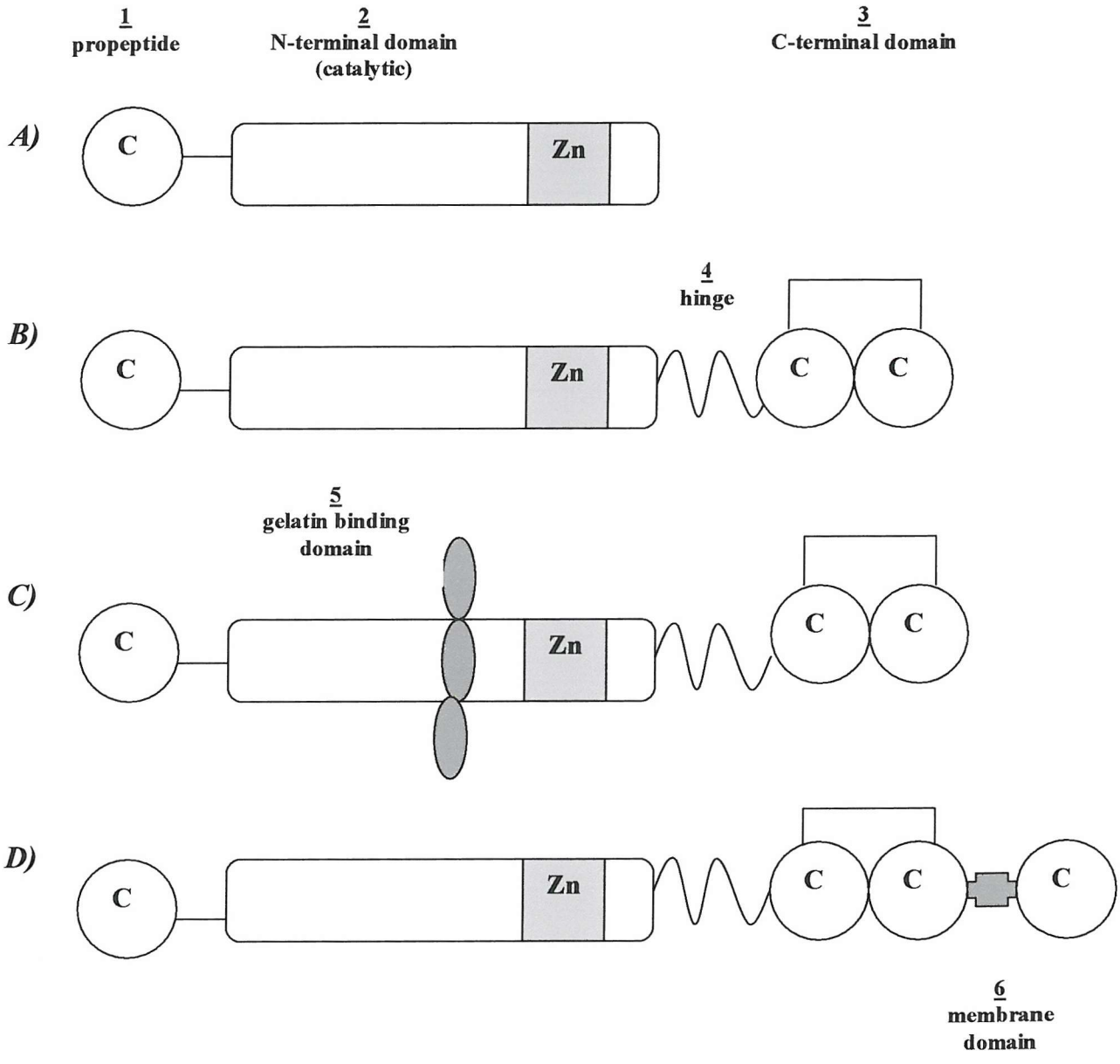
Three collagenases have been identified that can degrade the fibrillar collagens (types I, II and III). They are a 55 Kda enzyme MMP-1 which is synthesised and secreted by connective tissue cells and macrophages; a 75 Kda enzyme, MMP-7which is packaged in specific granules of polymorphonuclear leucocytes; and a 65 Kda enzyme MMP-13 which is found in certain human tumours (Freije et al, 1994).

Table 1.IV. Classes and properties of MMP's

	MMP numbering	**Mol Wt (latent)**	**Mol Wt (active)**	**Degraded substrate**
Collagenases	**MMP-1**	55kDa	45kDa	Fibrillar collagen types I,II,III,VII,VIII,X
	MMP-8	75kDa	58kDa	
	MMP-13	65kDa		Proteoglycan core protein
Gelatinases	**MMP-2**	72kDa	66kDa	Peptides from denatured collagen
	MMP-9	92kDa	86kDa	Gelatins, Elastin
Stromelysins	**MMP-3**	57kDa	45kDa	Proteoglycan core protein
	MMP-10	57kDa	44kDa	Type IX and X collagen, laminin
	MMP-11	51kDa		Elastin, fibronectin, Procollagens I, II II
Matrilysin	**MMP-7**	28kDa	19kDa	As for stromolysins
Metallo-elastase	**MMP-12**			As above
Membrane	**MMP-14**			Pro-MMP-2

The gelatinases are responsible for the further degradation of denatured collagen fibres by cleaving near their cross-link sites. The two gelatinases that have been identified are a 72 Kda form (Gelatinase A: MMP-2) derived from mesenchymal cells and a 95 Kda form (gelatinase B: MMP-9) associated with polymorphonuclear leucocytes, macrophages as well as some tumour cells and stimulated connective tissue cells. The combined action of the collagenases and the gelatinases is capable of degrading all components of the extracellular matrix. Further types of MMPs are being recognised including membrane bound MMP's which have a trans membranous domain, and may be involved in regulation of other MMP's and their activity.

Figure 1.8 Diagrammatic representation of domains within the MMP family



A) matrilysin

B) collagenases, stromelysins, metalloelastase

C) gelatinases

D) membrane type metalloproteinases

1.8 Regulation of MMP activity

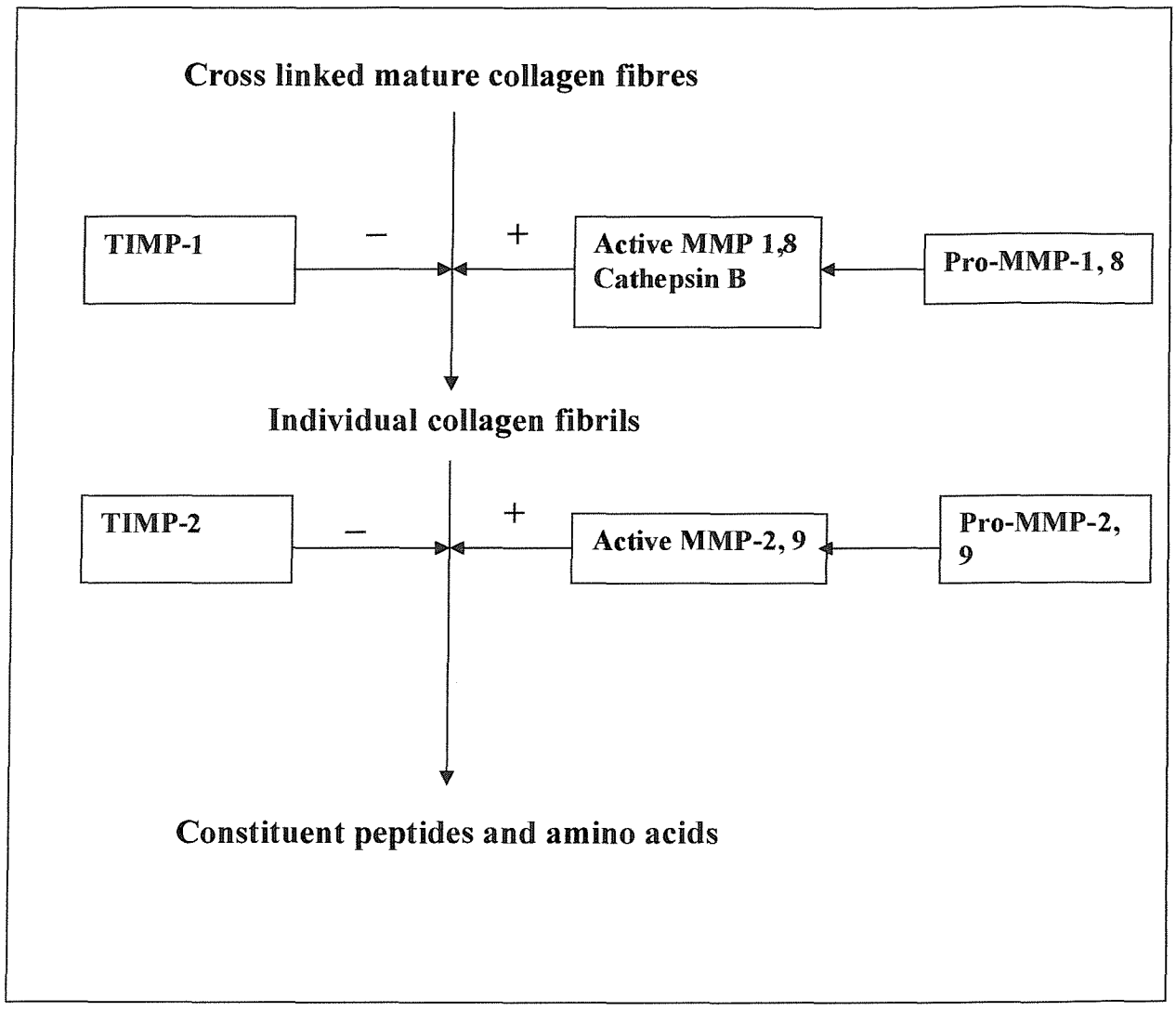
MMP's are potent enzymes and so have to be carefully regulated. This is achieved by three mechanisms: modulation of proenzyme production, activation of proenzyme and production of endogenous inhibitors (see figure 1.9). Firstly, MMP's are produced in a proenzyme form. Activation is achieved by the proteolytic cleavage of a peptide blocking the active site (Kleiner et al, 1993). This allows the zinc present at the active site to hydrolyse susceptible bonds in protein substrates. Increased production of the proenzyme allows greater concentrations available for activation. The regulatory pathways for MMP activation are unclear. However, plasmin, stromelysins and other MMP's can activate various members of the MMP family, and cytokines such as IL-1 (West- Mays et al, 1995), tumour necrosis factor alpha (TNF-α) (Mann et al, 1995) and plasminogen activator (Collier et al, 1988; Gavrilovic et al, 1989) have been shown to promote MMP activity in various tissues and in vitro systems. Changes in cytokine activity may play a key role in tissue turnover and degradation in various disease processes (Reis et al, 1995).

Once activated all MMP's are inhibited by local acting tissue inhibitors of metalloproteinases (TIMPs) which are present in all connective tissues (Denhardt et al, 1993) and bind to the MMP molecules in a 1:1 ratio. TIMP-1 is a 30kDa glycoprotein synthesised and secreted by most connective tissue cells, as well as macrophages, and can be identified in most bodily fluids. The amino acid sequence of TIMP-1 is again largely conserved across species and forms high affinity, irreversible, non-covalent bonds predominantly with MMP-s 1 and 8. The inhibitory activity of TIMP-1 has been shown to

reside in the first three loops of a structure defined by six disulphide bridges (O'Shea et al, 1992). TIMP-2 is a 21kDa unglycosylated protein which retains the six disulphide bridge domain seen in TIMP-1 but which mainly binds with the pro and active forms MMP-2. TIMP-3 has recently been identified in human tissues. It has a considerably different structure toTIMP's 1 and 2, yet still conserves the six disulphide bridge domain (Apte et al, 1995). Interleukin 10 (IL-10) and transforming growth factor β (TGF-β) are both able to modulate the synthesis of MMP's (down regulate) and TIMP-1 (up regulate) (Lacraz et al, 1995; Edwards et al, 1987).

The interactions between the pro-MMP formation, active MMP and TIMP are outlined in figure 1.9. An imbalance of proteases over inhibitors occurs both in physiological and pathological processes. It is now clear that no one proteinase can be considered of overiding importance in matrix turnover and that a complex cascade of proteolytic events best explains how tissue destruction takes place. Disease processes may result from an imbalance of MMP's over TIMP's such as seen in rheumatoid disease or tumour spread (Martell-Pelletier et al, 1994).

Figure 1.9 Regulation of MMP activity



1.9 Other components of the extracellular matrix

The connective tissues join the other tissues of the body together. They take the stress of movement, maintain shape and are made up of a composite of *insoluble fibres* and *soluble polymers*, all of which go to make up the extracellular matrix. The principal fibrous component is *collagen*, which provides tissue strength and has been discussed earlier. The other main fibrous component of the extracellular matrix is *elastin*, whilst the soluble polymers include *proteoglycans* and *glycoproteins*. The structure and function of a tissue depends on the relative proportions of each of these constituents. Hence a tissue requiring high tensile strength would have a high collagen content, whereas a tissue designed to withstand compressive forces (such as cartilage) would be rich in proteoglycans.

Elastic fibres

Elastic fibres give tissue the ability to retain its shape having been distorted by movement and consist of two protein components; the more abundant *elastin*, and an *elastic microfibril*, which is composed of a specialised glycoprotein. Mature elastin fibres are made up of individual polypeptide chains, called tropoelastin, which are covalently connected by the cross-links desmosine and isodesmosine. Elastin has a long half-life, and is degraded by the enzyme called elastase. Excess elastase may be involved in some diseases such as emphysema where the lung loses its elasticity.

Proteoglycans

The proteoglycans are a diverse family of molecules characterised by a core protein to which is attached one or more glycosaminoglycan (GAG) side chains. The major functions of the proteoglycans are to regulate the connective tissue structure, hydration and resist compressive forces, but they are also involved in regulating the permeability of the extracellular matrix to certain substances. Proteoglycans are diverse in structure and function. Highly acidic and hydrophilic GAG chains will have a major influence on tissue hydration and elasticity (Gallagher et al, 1986). The proteoglycans have a heterogeneity in structure due to variation in the GAG side chains, but retain similarity in their protein core. The glycosaminoglycan group of complex carbohydrates include chondroitin sulphate, dermatin sulphate, keratin sulphate, heparin sulphate and hyaluronan (hyaluronic acid).

Glycoproteins

Whilst the simple picture of the extracellular matrix explains how the collagen fibres resist tensile forces and the proteoglycans allow the tissues to swell with water and resist compressive forces, there are many other glycoproteins present within the extracellular matrix that play other important physiological roles. Many of the glycoproteins contain distinct and functionally active peptide domains that can interact with cell surface receptors as well as other matrix molecules. For example, in bone these proteins may interact with the inorganic phase of bone. The heterogenous glycoprotein group collectively contains carbohydrate covalently attached to the protein core. The two most studied examples of glycoproteins are *fibronectin* and *laminin*.

Fibronectin is a large (440,000 dalton) multifunctional glycoprotein consisting of two dimers held together by disulphide bonds. Fibronectin is produced by fibroblasts, monocytes, endothelial cells and other connective tissue cells. Fibronectins possess numerous repeating functional domains, which give them the important characteristic of binding both to macromolecules of the extracellular matrix such as collagens, fibrin and proteoglycans, as well as to cells surface integrin receptors. For this reason it is thought that fibronectin is involved in interactions (eg. attachment and spreading) of cells with the extracellular matrix (Hynes et al, 1995). It is thought that fibronectin may play a role in cell migration and tissue organisation in healing wounds.

Laminin is the most abundant glycoprotein found in basement membranes. It is a large glycoprotein (800,000 to 1,000,000-Da) adopting a cross-shape structure. The molecules span the basement membrane and bind both to specific receptors on the surface of cells, as well as matrix components associated with the basement membrane, such as collagen type IV and heparin sulphate. Laminin is believed to mediate cell attachment to the connective tissue and in culture can alter growth, survival, morphology, differentiation and motility of various cell types such as endothelial cell and neurites (Engvall, 1994).

Section 3: Collagen metabolism in pelvic organ prolapse

There is substantial evidence to suggest pelvic organ prolapse and genuine stress incontinence are attributed to direct muscular injury and denervation occurring as a result of childbirth. However, this does not explain why some women with normal neuromuscular function still develop genitourinary prolapse, nor why prolapse occurs in two per cent of nulliparous women. Norton et al (1990), suggested prolapse may be associated with abnormalities in the formation of collagen. Abnormalities of collagen encompass a clinical spectrum from joint hypermobility to the severe forms of Ehlers-Danlos syndrome and other connective tissue disorders. Norton et al (1990), assessed whether there was a relationship between joint hypermobility, (which can be clinically scored) and genitourinary prolapse, by studying a population of 107 women attending a general gynaecology clinic. They demonstrated a significantly higher incidence of pelvic relaxation in women with hypermobile joints compared with women with no joint laxity, and concluded that an underlying connective tissue abnormality may be a factor in the aetiology of pelvic organ prolapse. Furthermore women suffering with a connective tissue disorder such as Ehlers Danlos syndrome and Marfan's syndrome often complain of prolapse (McIntosh et al, 1995, 1996).

Makinen et al, (1986), studied the histological appearance of pre-cervical vaginal fascia in 10 women with significant uterine prolapse compared with 10 controls with no prolapse, and showed abnormal histological changes in the fascia of 7 out of the 10 women with prolapse compared with only 2 controls. The abnormal histological findings consisted of

an increase in the number of collagen fibres and a reduction in the fibroblast number. The group suggested that an abnormality in fibroblast function and collagen production may lead to the development of prolapse. However, later studies were unable to demonstrate any difference in the rate or quality of in vitro collagen produced by cultured fibroblasts taken from 5 women with prolapse compared with those cultured from 5 controls. (Makinen et al, 1987).

Genuine stress incontinence often co-exists with prolapse and the two are thought to have similar aetiologies. As mentioned earlier, the proportion of hydroxyproline in collagen remains constant, and can be measured to estimate the collagen content in a tissue. Ulmsten (1987), used the hydroxyproline assay to gain an estimate of total tissue collagen content of tissue taken from women with genuine stress incontinence and controls. They showed a reduction in the total collagen content of both vaginal skin and ligamentum rotundum in 7 women with genuine stress incontinence (GSI), compared with controls. This work suggested the deterioration in the connective tissue of these women was associated with the development of stress incontinence. Rechberger et al (1993) showed a reduction in collagen content in biopsies from the vesicovaginal junction in women with stress incontinence. They found this was associated with an increase in the concentration of oestrogen receptors in the vaginal skin biopsies. It is interesting to note that all the women in the study also had a cystocele that required repair. Therefore, as they all had prolapse, it makes it difficult to suggest that decreased collagen content can be attributed to the development of GSI alone in the absence of prolapse.

Norton et al (1996), studied the relationship of type I:type III collagen in vaginal fascial fibroblast cultures and showed a significantly reduced production of type I compared with type III collagen in fibroblast cultures taken from women with genital prolapse compared with controls ($P<0.0001$). They later showed a reduction in the type I:type III collagen ratio in biopsies of vaginal fascia taken from women with cystocele compared with controls (Norton et al, 1996).

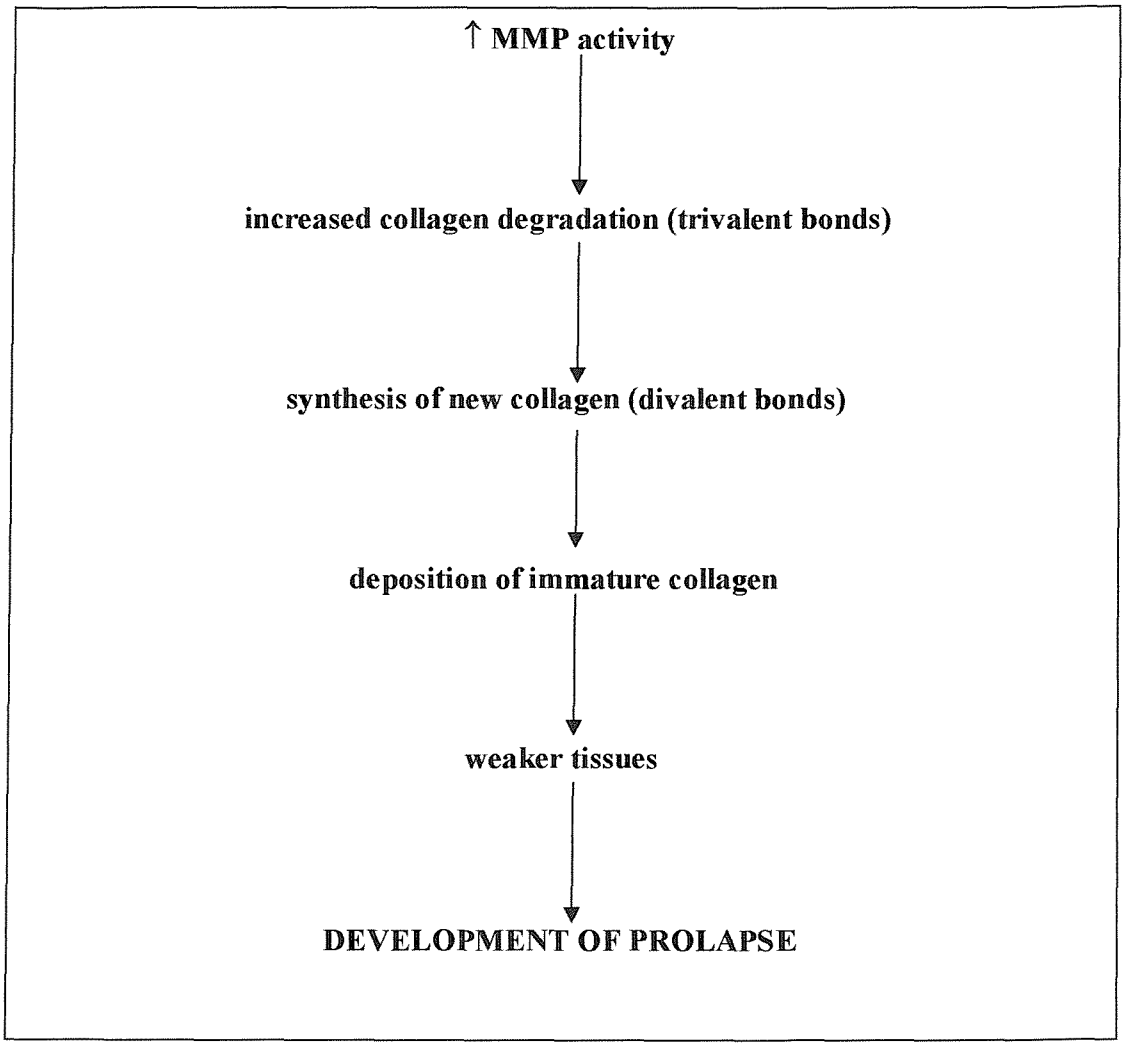
Keane et al (1997), analysed the hydroxyproline content, collagen types and collagen cross-links in nulliparous women with genuine stress incontinence compared with controls. Choosing a nulliparous population, they excluded any deterioration caused by childbirth. They showed a reduction in the hydroxyproline content of periurethral vaginal skin biopsies taken from 36 premenopausal nulliparous women with genuine stress incontinence ($p<0.0001$) compared with 25 controls. They also showed a significant reduction in collagen cross-linking in women with genuine stress incontinence ($p<0.0001$) along with a decreased ratio of type I to type III collagen ($p=0.0008$). Collagen type III is considerably more flexible and less resistant to tensile stress than type I collagen. The group suggested there was not only a deterioration in the quantity of collagen in women with genuine stress incontinence, but also in the quality of the connective tissue, reflected in a decrease in type I:type III collagen ratio, which both led to a reduction in tissue strength. As collagen III is more flexible and less resistant to strain than type I collagen, the group suggested the alteration in ratio of collagen types may have caused the tissues weaker properties and led to stress incontinence.

However, an alteration in the ratio of collagen typeI:III, in prolapse tissue has not been seen by other workers (Bergman et al, 1994, Jackson et al, 1996). The discrepancy in results may be explained by the methodology used to degrade the collagen from tissue. Cyanogen bromide digestion is often used to break the inter-fibrillar bonds of collagen. The peptides are then separated by SDS-PAGE electrophoresis and the bands measured by scanning densitometry of the gels. However, it is becoming increasingly apparent that as tissue ages the bonds become more resistant to cyanogen bromide digestion and so less collagen is extracted from the tissue and so it is difficult to draw any conclusions from such data (personal communication with N. Avery, Bristol Collagen Group).

Sayer et al, (1990) showed no difference in the total collagen content of pubocervical fascia taken from women with bladder neck prolapse compared with controls, but did note a change in the nature of the collagen. They found tissue from women with prolapse had significantly greater autofluorescence than controls ($p<0.001$). Sayer suggested that this may be attributed to differences in the cross-linking of the collagen fibrils. Cross-linking was further investigated by Jackson et al (1996), who examined vaginal fascia taken from the pericervical cuff of 8 women with uterine prolapse and associated cystocele and 10 women without uterine prolapse or cystocele. Samples were analyzed for total collagen content, intermolecular cross-links, collagen type I/III ratios and matrix metalloproteinase activity. They showed that in the vaginal fascia of women with uterine prolapse and cystocele there was a significant reduction in the total collagen content ($p=0.003$), along with an increase in the intermediate intermolecular cross-links hydroxylysino-keto-norleucine (HLKNL). This was associated with a rise in both the pro and active forms of

matrix metalloproteinase 2 (MMP-2) (p=0.002 and 0.048 respectively) and the pro form of MMP-9 (p=0.048). They did not show any difference in the ratio of collagen type I to collagen type III. Further work by James et al (1999) has confirmed these findings with a larger study population. These workers have suggested genitourinary prolapse occurs in individuals as a result of an increased degradation of collagen caused by inherent elevated levels of matrix metalloproteinases. The degraded collagen is then replaced by newly synthesised immature collagen, which has weaker divalent intermediate cross-links which is more susceptible to degradation. The combination of immature collagen and a reduction in the total collagen content results in weakness within the tissue and hence leads to prolapse (see figure 1.10). The group also suggested that manipulation of collagenolytic activity may hold therapeutic possibilities, such as using steroid hormones to regulate proteinase gene expression.

Figure 1.10 Hypothesis for aetiology of prolapse based on work by Jackson et al, 1996.



Section 4: Oestrogen receptor and smooth muscle phenotype

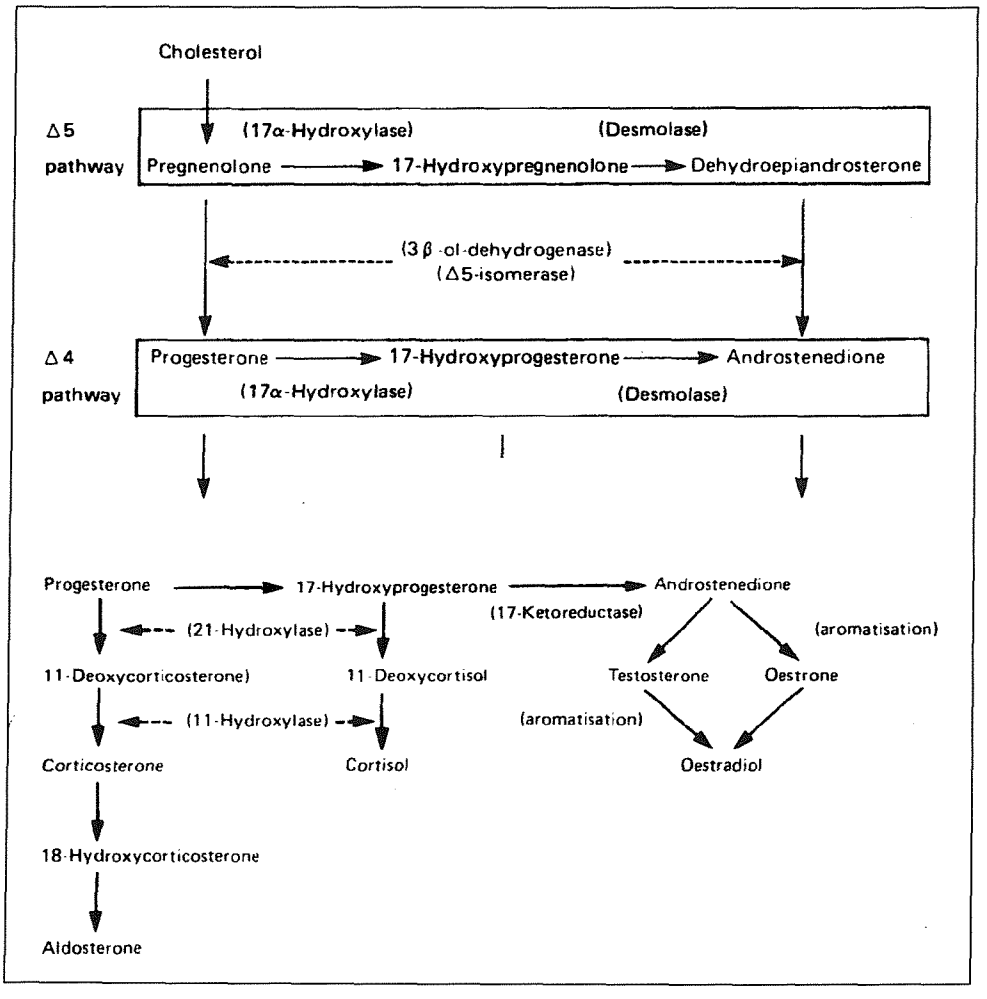
1.10 Oestrogen

An oestrogen is a sex steroid hormone synthesised in vivo from cholesterol (see Fig.1.11), with oestradiol being the major oestrogen in the non-pregnant premenopausal woman. Oestrogen has many effects on the development of secondary sexual characteristics and maturation of the female genital tract and pelvis, along with regulating menstruation, ovulation and pregnancy. In addition oestrogen affects metabolic changes on bone mineralisation, lipid metabolism, coagulation and cardiovascular physiology. Oestrogens are also formed in the peripheral tissues, particularly skin and adipose tissues, by the conversion of androstenedione to oestrone, which is the major endogenous source of oestrogen in the postmenopausal female (Chard et al, 1995). However, once ovarian function has declined, this is not sufficient to maintain premenopausal physiological levels of circulating oestrogens. Oestrogen deficiency, seen after the menopause therefore induces metabolic and trophic changes. Early symptoms of the menopause include vasomotor instability (causing hot flushes), and sleep disturbances (Rebar et al, 1987; Schiff et al, 1980). Later mood changes and depression may develop (Bungay et al, 1980) and atrophic changes become evident resulting in vaginal dryness, itching, discomfort and dyspareunia (Hammond 1996). Elevated demineralisation of bone is associated with osteoporosis and an increased risk of bone fracture (Christiansen et al, 1980) and postmenopausal women approach male-specific morbidity and mortality from

cardiovascular disease due to changes in lipoprotein pattern and direct effects on blood vessels (Jensen et al, 1986; Hillard et al, 1991).

Prolapse and urinary incontinence are also associated with the postmenopause and it is thought that oestrogen deficiency may be an important factor in their development. The sites for action of oestrogen are summarized in figure 1.12.

Figure 1.11 In vivo production of oestrodiol from cholesterol



1.11 The oestrogen receptor

Oestrogens, like other steroid hormones exert their effect via ligand-activated transcription factors, called oestrogen receptors (Enmark et al, 1999). The oestrogen receptor is part of a nuclear receptor superfamily with more than 70 members. Other members of this family include the receptors for progesterone, testosterone, corticoids, thyroid hormone and vitamins A and D. All the family of steroid hormone receptors possess similar functional domains (Evans 1988). There is a highly conserved DNA-binding domain containing two zinc fingers which are involved in specific chromatin recognition and DNA binding, and a less well conserved hormone-binding domain housing regions important for ligand binding, nuclear localisation and interactions with transcription co-activators and co-repressors (Kumar et al, 1987). The hormone binds to the receptor in the cytoplasm of the target cell and then the receptor-ligand complex migrates to the nucleus to bind to specific sequences in the DNA. This leads to transcription and synthesis of new proteins by the target cell to exert the effects of the hormone. It is therefore generally accepted that the presence of oestrogen receptors is a prerequisite for a target tissue to recognise oestrogen and to manifest the effects of the hormone.

A second subtype of the oestrogen receptor has been discovered and named the oestrogen receptor beta (Kulper et al, 1996, Enmark et al, 1997). However, to date there is little in the literature regarding distribution of each subtype of the receptor. Some work has shown an increase in the expression of oestrogen receptor beta mRNA in ovarian endometriomata (Fujimoto, et al, 1999). As to date there is little research into the

expression of the oestrogen receptor beta, further reference to the oestrogen receptor will be regarding the alpha subtype unless otherwise stipulated.

1.12 Target tissues for oestrogen

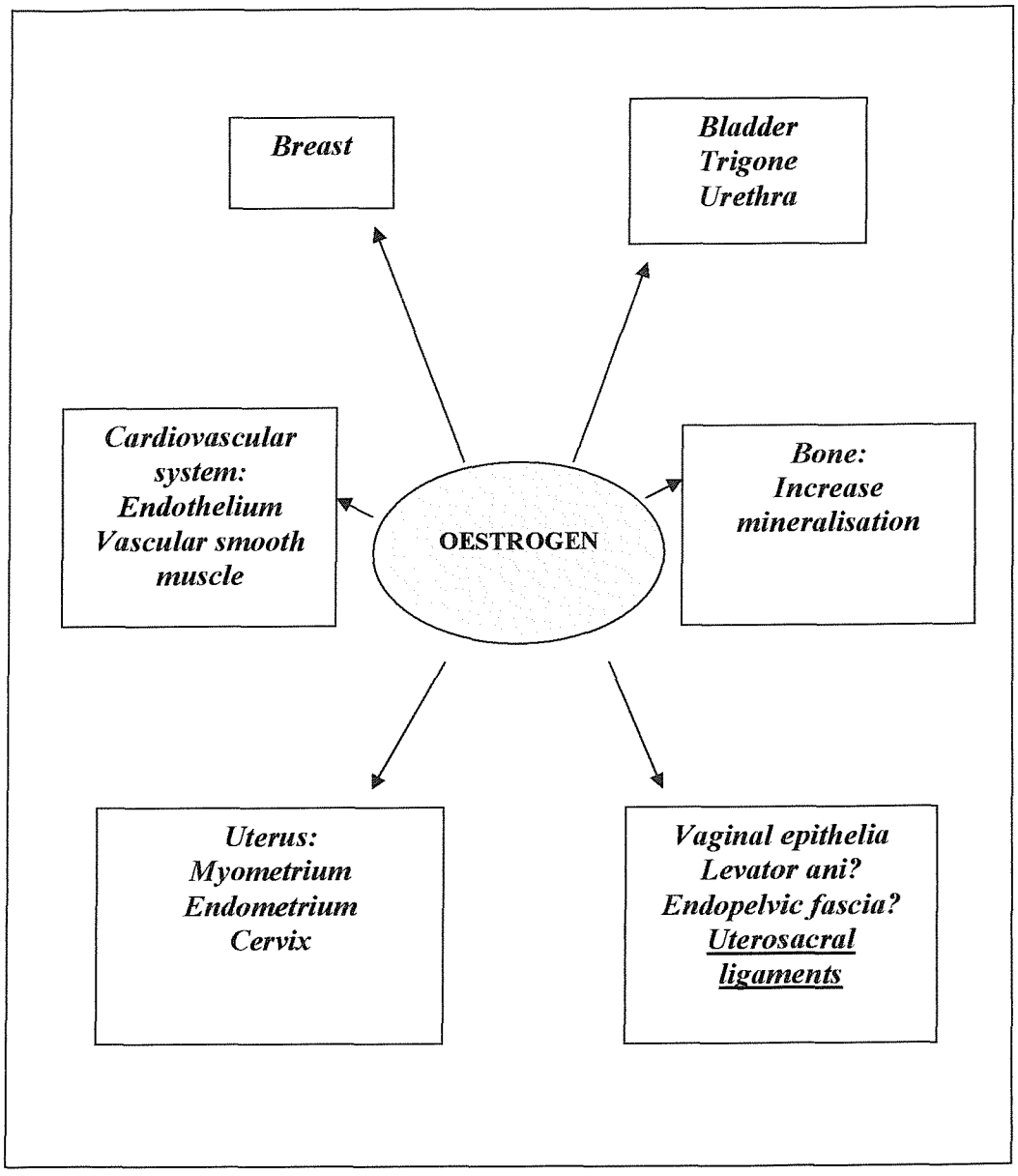
Press et al, (1986) demonstrated the presence of oestrogen receptors in the human vagina, uterus and fallopian tubes. The staining for the oestrogen receptor was entirely localised to the nucleus of epithelial cells, lamina propria, smooth muscle cells of the myometrium and stromal cells. They did comment that the intensity of staining of the oestrogen receptor in slides altered with the phase of the menstrual cycle. They noted more pronounced staining in the stratum functionalis of the endometrium than in the vaginal epithelium. The serosa of the genital tract, connective tissue and blood vessels and inflammatory cells did not stain for the oestrogen receptor.

Oestrogen receptors have also been demonstrated throughout the female lower urinary tract. Both human and animal studies have shown oestrogen receptors in the trigone and other areas of the urinary bladder as well as the female urethra, although at much lower concentrations than found in the uterus (Iosif et al, 1981; Bussolati et al, 1990; Batra et al, 1983). It is thought that oestrogen may play a role in maintaining a functional urethra by stimulating growth of the urethral epithelium (Bhatia et al, 1989; Bergman et al, 1990). Low dose oestrogen causes an increase in blood flow to the urogenital tissues which may aid epithelial growth and cause engorgement of the submucous venous plexus of the urethra (Batra et al, 1986; Rud et al, 1980).

Oestrogen may exert an affect on the supportive tissues of the urethra, bladder neck and pelvic organs thus helping to maintain continence mechanisms and preventing prolapse. Hilton (1983), showed the maximum urethral closure pressure on stress was significantly increased in women using intravaginal oestrogen cream, and concluded that the improved pressure transmission in the mid-urethra was attributed to effects of oestrogen on the pelvic floor. Several studies have shown oestrogen receptors in the levator ani muscle, round ligament and myometrium, although only at very low concentrations (Ingelmann-Sundberg et al, 1981; Smith et al, 1993). These studies mainly quantified oestrogen receptor concentration by ELISA techniques on pulverised biopsies of tissue. However, this technique prevents the exact histological localisation of the oestrogen receptor and samples may have become contaminated by surrounding connective tissue or fascia especially if the samples were taken vaginally. Immunostaining of frozen tissue from one study showed some staining for the oestrogen receptor in the cytosol of striated muscle cells of the levator ani muscle. However, the oestrogen receptor is a nuclear protein, synthesised in the cytoplasm and then rapidly transferred to the nucleus (King and Greene, 1984). Natural background staining of the cytoplasm can occur as part of the preparation process and may be misinterpreted as positive staining. Bernstein et al (1999), examined paraffin fixed biopsies of levator ani muscle stained with a monoclonal antibody specific for the oestrogen receptor. The immunostained sections were evaluated by registration of the percentage of positively stained nuclei in the striated muscle cells, thus avoiding contamination from other tissues. They showed there were no oestrogen receptors present in the nuclei of striated muscle cells in any of their specimens of levator ani muscle, nor in

the connective tissue cells within the striated muscles. It is therefore unlikely that the musculature of the pelvic floor is sensitive to oestrogen.

Figure 1.12: Sites of action of oestrogen



1.13 Effects of oestrogen on connective tissue

There is evidence to suggest that the connective tissue of the endopelvic fascia may respond to oestrogen. Brincat et al (1983, 87), showed that skin collagen content was lower in postmenopausal women not taking oestrogen replacement than postmenopausal women on hormone replacement therapy. Versi et al, (1988) showed a correlation between urethral pressure and skin collagen content and suggested that oestrogen may affect urethral function by changing collagen metabolism in the paraurethral tissues in a similar fashion as seen in the skin biopsies.

Administration of oestrogen may directly affect the composition of the extracellular matrix. Savvas et al (1993), showed that the administration of oestrogen to postmenopausal women resulted in an increase in the deposition of collagen type III found in skin biopsies taken from these women. It is questionable whether this would provide significant support and perhaps measuring the total collagen content may have been more appropriate. The composition of the extracellular matrix of the endometrium of postmenopausal women can be manipulated by the administration of equine oestrogen. Iwahashi et al (1997), showed that the distribution and proportion of collagen types I, III, IV, VI and laminin in the endometrium of women receiving oestrogen supplementation had an altered distribution and composition compared with controls. Although these data may not show that the administration of oestrogen will cause an increase in connective tissue collagen content or strength, they do demonstrate that the administration of oestrogen can affect the quality of the extracellular matrix.

During menstruation the extracellular matrix of the endometrium is lysed to allow shedding. Increased MMP-1 activity within the endometrium is seen in the perimenstrual phase of the menstrual cycle, coincident with a fall in endogenous oestrogens (Hampton et al, 1994). During this process the MMP-1 expression is predominantly localised to the plane of tissue shedding (Kokorine et al, 1996). Singer et al (1997), found that endometrial explants released considerable MMP-1 when cultured in the absence of ovarian hormones. They further showed that the addition of ovarian hormones to the culture medium inhibited the release of interleukin-1and repressed MMP-1 production by endometrial explants. This may suggest that oestrogen mediates its affects on the extracellular matrix of the endometrium, by altering the expression of the matrix metalloproteinase enzymes from cells lying within the matrix.

1.14 Smooth muscle phenotype and function

If MMP expression is affected by the action of oestrogen, it would be of interest to determine which cells are responsible for MMP production within the endopelvic fascia of women with prolapse? It has already been shown that there is no alteration in fibroblast function within the connective tissue of women with prolapse, (Makinen et al, 1987) and so fibroblasts do not appear to be involved. Smooth muscle cells are a member of myofibroblast family and are able to undergo transition into myofibroblasts, and so may develop similar functions to fibroblasts. It has been shown that smooth muscle cells can produce MMP's and TIMP's to effect the extracellular matrix in *in vitro* conditions (Sansilvestri-Morel et al, 1998).

By studying the phenotype (antigen expression) of the smooth muscle cells it is possible to see if the smooth muscle cells have transformed into myofibroblasts. Ricciardelli et al (1989) showed that smooth muscle cells cultured from prostate explants underwent a transition from a smooth muscle morphology and phenotype into a myofibroblastic morphology and phenotype. During their transformation these cells showed a decline in the predominant smooth muscle cell marker "*desmin*". The cells also possessed increased content of rough endoplasmic reticulum indicating they had developed a secretory / protein synthetic capacity (Ricciardelli et al, 1989).

"*Vimentin*" has been described as the predominant antigen in the mesenchymal cells: myofibroblasts. However, both desmin and vimentin co-exist in smooth muscle and myofibroblasts, but in varying quantities. "*Alpha smooth muscle actin*" is also present in both cell types but can been in much higher concentrations in areas of tissue scarring, where myofibroblasts predominate (Muchaneta-Kubara et al, 1997).

Zhang et al (1997), developed an *in vitro* model for prostatic smooth muscle cells. They showed that the addition of oestradiol to the medium caused an increase in the growth of the smooth muscle cells measured by an increase in expression of the smooth muscle cell markers "*desmin*" and "*smooth muscle myosin*". These studies show that by observing the phenotype of smooth muscle cells and myofibroblasts, one can see a transition from one cell type to the other. Previous studies have looked at the effect of the administration of oestrogen to cultured cell lines of smooth muscle cells and shown a response by cultured cells. This response must have been mediated via an oestrogen receptor. To date there

have been no studies to quantify oestrogen receptor expression in the uterosacral ligaments from women with prolapse. Moreover, no studies have looked for a relationship between oestrogen receptor expression and smooth muscle cell / myofibroblast phenotype in the uterosacral ligaments of women with prolapse.

Section 5: HYPOTHESES AND OBJECTIVES

The majority of work to date has examined collagen metabolism in tissue encompassing vaginal skin and the layers of fascia in close proximity to the vaginal skin. This is because it is more convenient to take samples of this tissue and these sites show demonstrable signs of prolapse. However, as discussed in Section 3 of this chapter, the vaginal skin is not an important supporting structure. Previous studies have demonstrated that the uterosacral ligaments and the fascia of the paracolpium are the predominant supportive structures (Mengert et al, 1936; DeLancey 1993).

Therefore, it is of interest to investigate if similar changes in collagenolytic activity and collagen content found in the vaginal skin (Jackson et al, 1996) also occur in the uterosacral ligaments.

Primary hypotheses:

The hypothesis to be tested is that there is an elevation in matrix metalloproteinase expression in the uterosacral ligaments as well as in the vaginal tissue of women with uterine prolapse. In addition, there are relationships between markers of collagen metabolism and collagen content in the two tissues signifying that the vaginal skin is reflecting changes in the uterosacral ligaments.

Oestrogen has been shown to influence MMP activity, collagen content and collagenolytic activity. Prolapse is common after the menopause and so the aetiology of prolapse may be linked to altered collagenase activity mediated as a result of reduced circulating oestrogen or altered oestrogen receptor expression in the smooth muscle of the uterosacral ligaments. In addition, smooth muscle cells have the ability to change their phenotype to myofibroblasts and in doing so alter their function, which could occur in prolapse tissue.

Secondary hypotheses:

Oestrogen receptor expression within the smooth muscle of the uterosacral ligaments differs between the premenopausal and postmenopausal states. In addition oestrogen receptor expression and smooth muscle cell phenotype are different in the uterosacral ligaments of women with prolapse compared with controls.

METHODOLOGY

Chapter 2:

Tissue sampling

Contents to Chapter 2:

Chapter 2: Tissue sampling

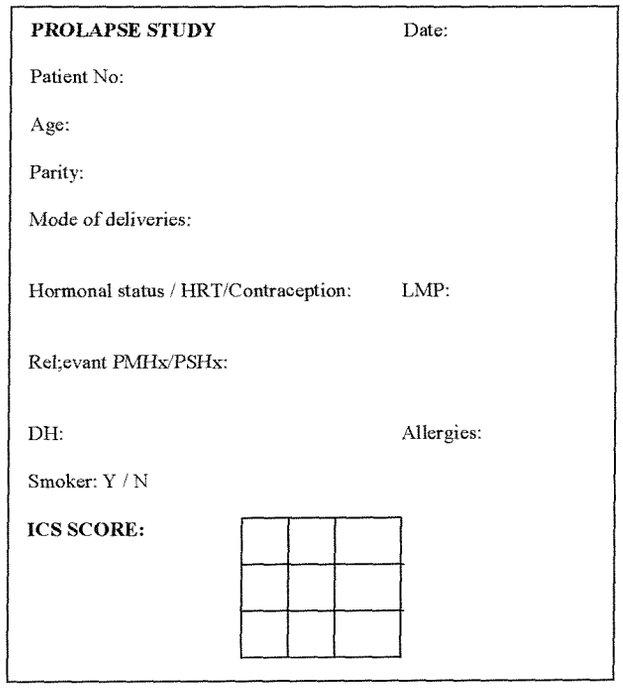
2.1 Ethical approval

Outlines of the project design were approved by the Southampton and South West Local Research and Ethics Committees, submission number 074/98.

2.2 Patient consent

Women awaiting hysterectomy for benign conditions including prolapse were asked if they would consent to be included in the study. If they consented a brief history was taken. The age, hormonal status and last menstrual period were noted for each patient. The duration of use of hormonal replacement therapy, GnRH analoques and contraception were also recorded where applicable. A past obstetric and medical history were obtained along with a note of any medication taken by the patient. Allergies and their smoking status were also noted. Patients were asked if they would consent to a vaginal examination and pelvic assessment was performed in accordance with the International Continence Society Pelvic Organ Prolapse Classification (outlined below). All data for each patient were recorded on a sheet similar to that illustrated in figure 2.1.

Figure 2.1 Data collection of Patient Details



2.3 Exclusion criteria

Patients were excluded from the study if they had any of the following conditions:

Pelvic malignancy
Fibroids
Endometriosis: active or old
History of pelvic inflammatory disease / previous positive chlamydia swab
Previous pelvic surgery

This was due to the effects the above conditions have on collagen metabolism and matrix metalloproteinase activity. Tissue was only obtained from patients who gave their consent for tissue samples to be removed and stored after surgery.

2.4 Power calculation

Based on the work by Jackson et al, (1996) a power calculation was performed. In their study the least difference between prolapse and control tissue was seen in the expression of active MMP-2 and so this was the parameter used for the power calculation. The difference in active MMP-2 expression was 120 units with a standard deviation of 85 units. The standardized difference was calculated as 120/85 = 1.4. The power for this study was set to be 0.9 (90%) with a 1% significance level. Using a nomogram for calculating sample size and power (Altman, 1995) the total sample size needed was 24.

2.5 Classification of prolapse

Until recently there has been little scientific study of prolapse due to the lack of a validated, objective and acceptable method for reproducibly describing the spectrum of pelvic support and degree of prolapse in individual patients and study populations. In 1995 the International Continence Society (ICS) devised a site-specific quantitative description of support that locates six defined points around the vagina (two anterior, two posterior and two apical) with respect to their relationship to the hymen (Bump et al, 1996). Points above the hymen are assigned a negative number and points distal to the hymen are assigned a positive number, with the plane of the hymen being defined as zero (0). For example, a cervix that protrudes 3cm distal to the hymen would be +3cm. The six points measured are:

Aa is the point in the midline on the anterior vaginal wall 3 centimetres proximal to the external urethral meatus. By definition, the range of position of point Aa relative to the hymen is –3 to +3cm.

Ba is the most distal position of the remainder of the anterior vaginal wall.

C is the most distal edge of the cervix or the vaginal cuff scar.

D is the location of the posterior fornix if the patient has a cervix.

Ap is the point in the midline on the posterior vaginal wall 3 centimetres proximal to the hymen. By definition, the range of position of point Ap relative to the hymen is –3 to +3cm.

Bp is the most distal position of the remainder of the posterior vaginal wall.

In addition the system requires three further measurements:

Gh (genital hiatus) is measured from the middle of the external urethral meatus to the posterior midline portion of the hymen.

Pb (perineal body) is measured from the posterior margin of the genital hiatus to the middle of the anal opening.

Tvl (total vaginal length) is the depth of the vagina when point C/D is reduced to is normal position.

All points except the total vaginal length are measured with the patient performing the valsalva manoevre. Points C/D are measured using a Cuscoe's speculum and forceps with 1 centimetre graduations marked along its length (see figure 2.2). Points Aa, Ab, Pa, Pb are measured using a Sim's speculum to retract the anterior or posterior vaginal wall.

Once measurements have been made patients are assigned to one of five ordinal stages according to the most severe portion of their prolapse. The stages are as follows;

Stage 0 no prolapse is demonstrated, ie. All points are at their highest possible level above the hymen.

Stage 1 the criteria for Stage 0 are not met, but the most distal portion of the prolapse is more than 1 centimetre above the level of the hymen.

Stage 2 the most distal portion of the prolapse is 1 centimetre or less proximal to or distal to the plane of the hymen.

Stage 3 the most distal portion of the prolapse is more than 1 centimetre below the plane of the hymen but protrudes no further than 2 centimetres less than the total vaginal length in centimetres.

Stage 4 essentially complete eversion of the total length of the lower genital tract is demonstrated.

The ICS POP Quantitation and Staging system is a useful tool for providing a summary description of a study population. The nine-point system is useful in describing pelvic support in a specific patient from one clinician to another. It is also helpful in following prolapse in an individual patient through time or comparing the effects of surgery. The ICS POP Q Classification has been standardised and reproducibility studies have documented both the inter- and intra-observer reproducibility (Hall et al, 1996). Examples of the ICS POP Q classification from a patient with normal pelvic support and complete vaginal eversion are seen in figures 2.4 and 2.5 respectively.

Figure 2.2 ***Instruments used in performing the ICS pelvic organ prolapse quantitation of prolapse system. Cuscoes bivalve speculum ©, Sim's speculum (S) and graduated forceps with 1 cm graduations (F).***

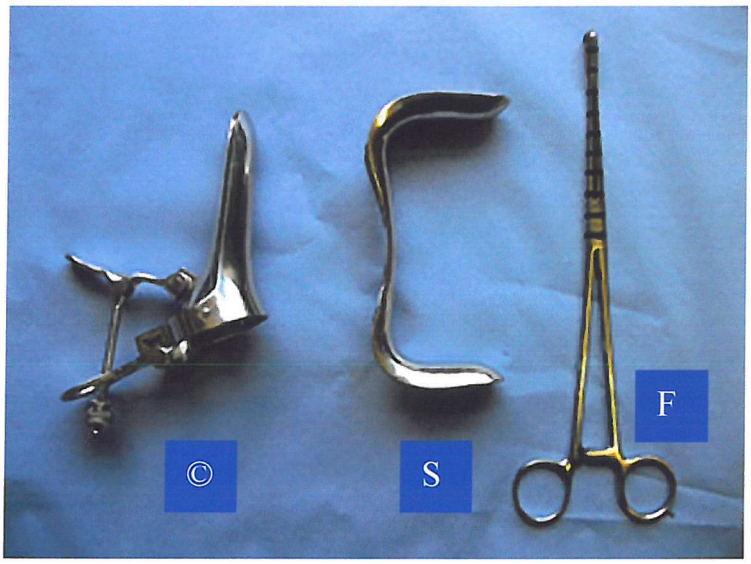
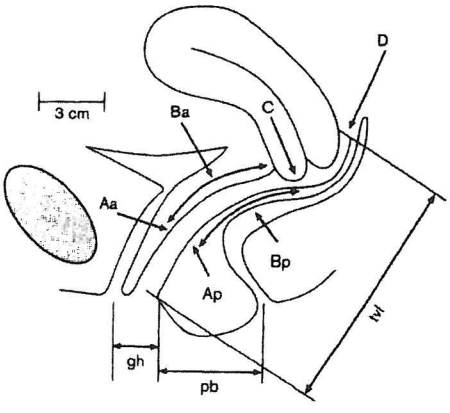


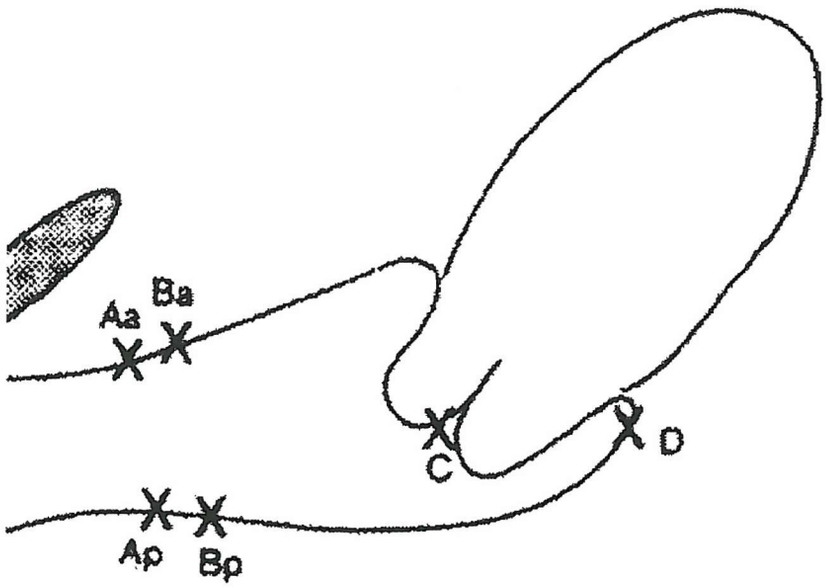
Figure 2.3 ***The six sites (Aa, Ba, C, D, Bp and Ap), the genital hiatus (gh), perineal bod (pb) and total vaginal length (tvl) used in pelvic organ support classification. These are then recorded in a three-by-three grid.***



anterior wall **Aa**	anterior wall **Ba**	cervix or cuff **C**
genital hiatus **gh**	perineal body **pb**	total vaginal length **tvl**
posterior wall **Ap**	posterior wall **Bp**	posterior fornix **D**

Figure 2.4 ***An example of a patient with normal support.***

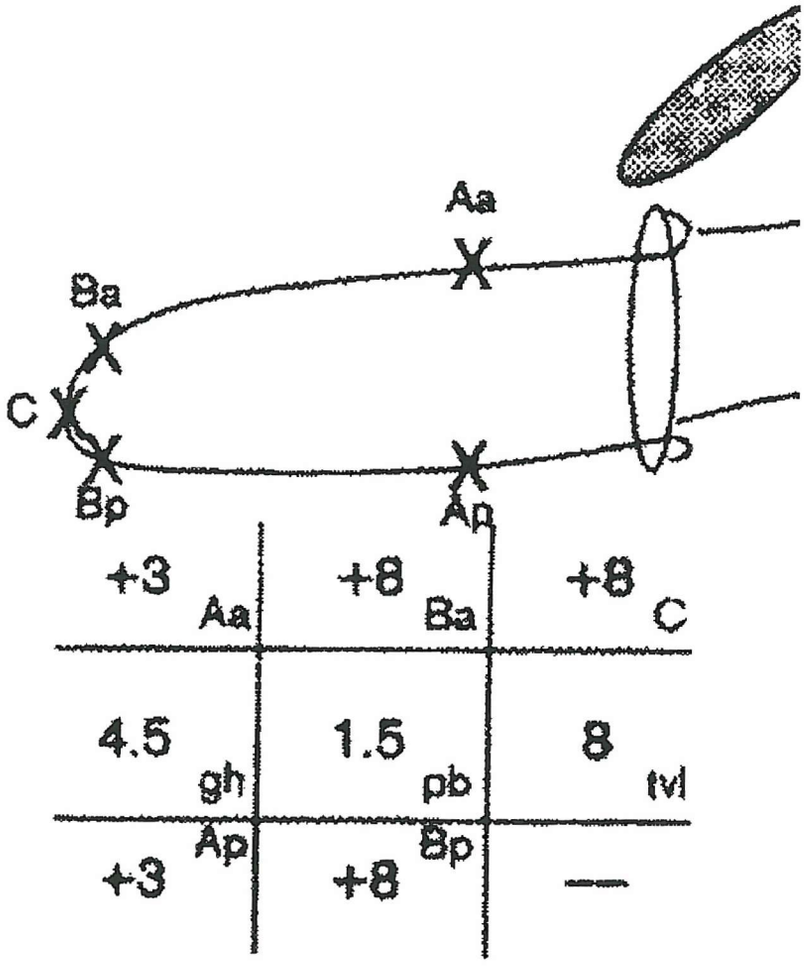
Grid and line diagram of the above patient: points Aa and Ba and points Ap and Bp are all –3 since there is no anterior or posterior wall descent. The lowest point of the cervix is 8 cm above the hymen (-8) and the posterior fornix is (-10). The vaginal length is 10cm and the genital hiatus and perineal body measure 2 and 3cm respectively. This represents Stage 0 support.



-3 Aa	-3 Ba	-8 C
2 gh	3 pb	10 tvl
-3 Ap	-3 Bp	-10 D

Figure 2.5 ***An example of a patient with procidentia of the uterus and complete eversion of the vagina.***

Grid and line diagram of the above patient. The most distal point of the anterior wall (point Ba), the cervix (point C0 and the most distal point of the posterior wall (point Bp) are all at the same point (+8). Points Aa and Ap are are maximally distal (both at +3). The fact that the total vaginal length equals the maximun protrusion makes this Stage 4 prolapse.



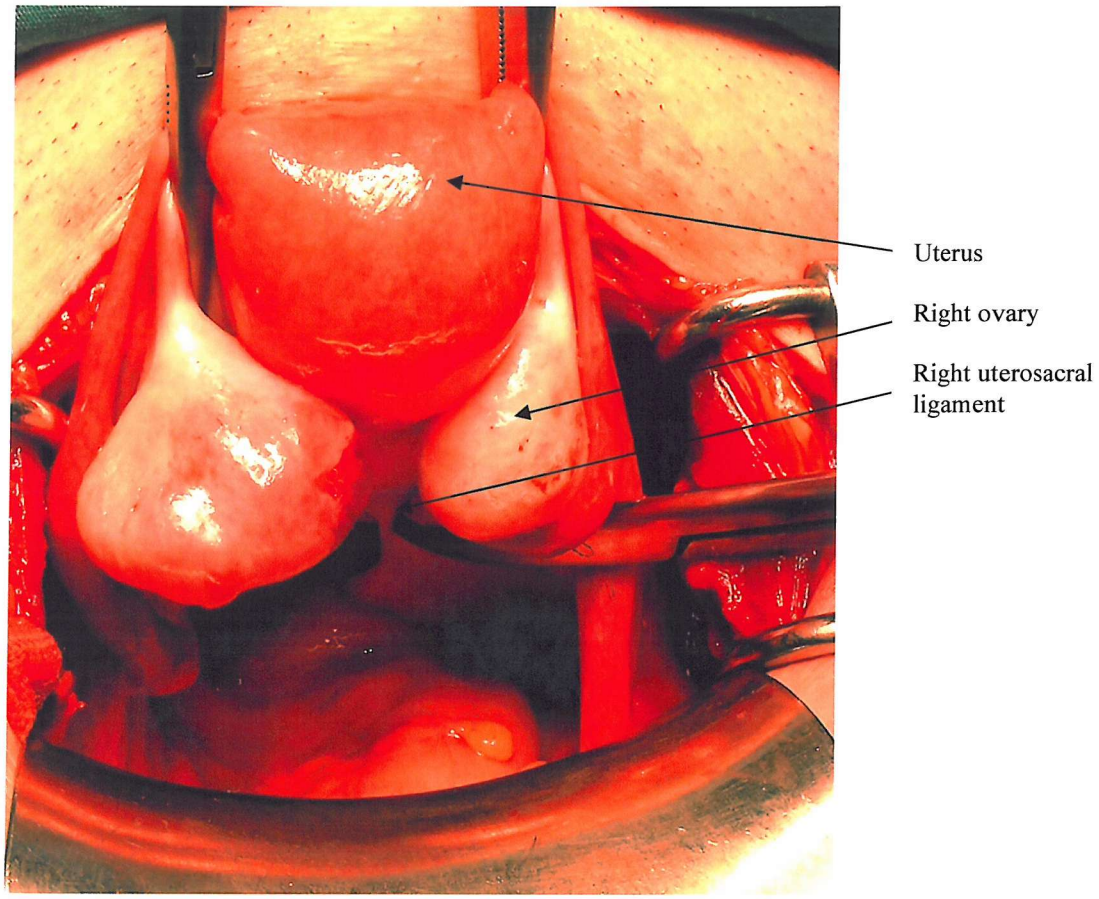
2.6 Harvesting of tissue and storage of tissue samples.

All women underwent their operations under general anaesthesia. All women having a hysterectomy for prolapse had the procedure performed vaginally. An abdominal procedure was performed for benign conditions where little / no prolapse existed. At operation tissue samples of the uterosacral ligament and the vaginal cuff were obtained.

The first biopsies were taken from both uterosacral ligaments 1 cm from its insertion into the uterus. This site was chosen for two main reasons. Firstly, Campbell (1950) noted from his histological evaluation of the uterosacral ligament that this would be the site along the uterosacral ligament most involved in supporting the uterus. Secondly it was easily accessible and did not require any alteration in surgical technique, which may have caused greater morbidity to the patient. These two biopsies (one left and one right uterosacral ligament) measured approximately 1x1cm in diameter. At hysterectomy the uterosacral ligaments are easily visible whilst performing the surgery however, once the uterus has been removed identification of these structures becomes more difficult as they retract into the substance of the paracolpium. To aid identification of the biopsy sites the uterosacral ligaments were marked 1cm from their insertion into the uterus with a marker suture of vicryl. Once the uterus was removed the sutures were separated and the tissue samples immediately taken. The area from which the first biopsy was taken is shown in figure 2.6. The second biopsy site was taken from the skin of the vaginal cuff in the midline 1 cm anterior to the cervix. This site is similar to that chosen by Jackson et al, (1996).

Biopsies were bisected and then one half of each tissue was placed in foil and immersed in liquid nitrogen. These samples were then stored at –80°c and later processed and analysed for collagen metabolism. The other half of the biopsies were immersed in 10% buffered formalin. These were later processed for histological examination.

Figure 2.6 ***Diagram illustrating the site from which biopsy-1: the uterosacral ligament were taken. This site was marked with a vicryl suture at operation to help identification once the uterus was removed.***



METHODOLOGY

Chapter 3:

Assays related to collagen metabolism

Contents to Chapter 3

Chapter 3: Assays related to collagen metabolism

3.1 Techniques for homogenising tissue samples

Prior to analysing biopsies from the study population, excess tissue was harvested and pooled to form a "stock" sample of tissue. This was then used to standardise working conditions for each laboratory technique.

Stock tissue was quickly blot dried and weighed. Samples of stock tissue were then immersed in Dulbecco's Phosphate Buffered Saline (PBS) at a concentration of 10mg tissue + 100µl of PBS (100mg/ml) and homogenised using either of two techniques:

1. Homogenisation of tissue with PBS, on ice for 4 x 7 seconds using a Labour technik T8.01 Ultra-Turrax homogenizer by IKA-WERKE, GMBH & Co.KG, (Staufen, Germany) at 13,000 rpm: the "*blender*" system (see figure 3.1).
2. Tissue was immersed in liquid nitrogen and freeze dried, then pulverised using a Mikro dismembranator by B. Brown Biotyech (Melsungen, Germany). The pulverised powder was then diluted with the appropriate volume of PBS: the *"dismembranator"* system (see figure 3.1).

After "blending" or "dismembranation" samples were placed in an IEC Centra-4x centrifuge, (Int. Equipment Company, Dunstable, England) and spun down for 60 seconds at 12,000 rpm. Supernatants were then taken for analysis.

The aim of initially using two techniques to homogenise tissue was to assess which technique extracted the most protein into the supernatant in addition to giving the most reproducible results. Samples of the stock tissue were taken and homogenised by each technique on four occasions. Serial dilutions of the supernatants were then made with PBS. After dilutions were made the amount of total protein extracted was determined against an albumin standard. By analysing samples four times the reproducibility of each homogenisation technique could be determined using the following equation:

$$\text{The coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100 = X\%$$

Serial dilutions were carried out to find the appropriate dilution of samples so that results fell on the linear portion of the standard curve.

Figure 3.1 ***Photograph illustrating the Mikro-dismembranator by B. Brown Biotech (TOP), the process involves freeze drying the tissue in liquid nitrogen and then pulverising the tissue into a powder. Ultra-Turrax T8 homogeniser (BOTTOM) purees tissue on ice.***

3.2 Measurement of total protein extracted from tissue.

The total protein extracted within the supernatants was measured in order to determine the following:

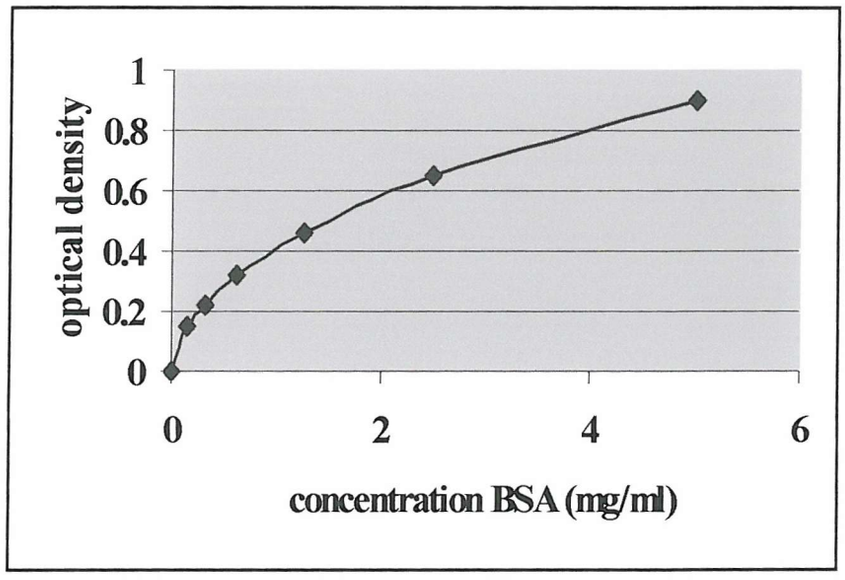
1. To check the reproducibility of homogenisation,
2. To calculate the amount of matrix metalloproteinase (MMP's) or tissue inhibitors of MMP's (TIMP's), relative to extracted material (protein in this case).. This prevents any inaccuracy in measuring the concentration of MMP's or TIMP's, which may have been introduced by the homogenisation technique alone. ie. on some occasions more protein may be extracted from a similar piece of tissue than on another occasion.

The protocol for measuring the total protein concentration was as follows:

1. Serial dilutions of bovine serum albumin (BSA) were used for the standards: 0mg/ml, 0.155mg/ml, 0.312 mg/ml, 0.625 mg/ml, 1.25mg/ml, 2.5mg/ml, 5mg/ml and 10mg/ml.
2. 250µl of Coomassie blue protein assay reagent (Pierce Cat No: 2320, Illinois, U.S.A.) were added to 50µl of the standards, controls and the samples to be analysed within a 96 well microplate.
3. All measurements were made in duplicate.
4. The plate was then placed on a plate shaker and left for 20 minutes. Wells were then analysed using a 96 well plate reader (Dynex Technologies MRX plate

reader, Dynex Technologies, Ashford, Middlesex, U.K.) measuring the optical density of wells using a test filter of 630nm and reference filter of 410nm (see figures 3.2 & 3.3).

Figure 3.2 Standard curve for total protein concentration.



The standard curve for bovine serum albumin, used as a standard for total protein measurements, is shown in figure 3.2. Samples of supernatant (concentration 100mg blot dried tissue / ml PBS) required a dilution x10 with PBS to lie within the central portion of the standard curve (ie. between optical densities 0.2 – 0.6).

Figure 3.3: Illustration of the Dynex Technologies MRX 96 well plate reader

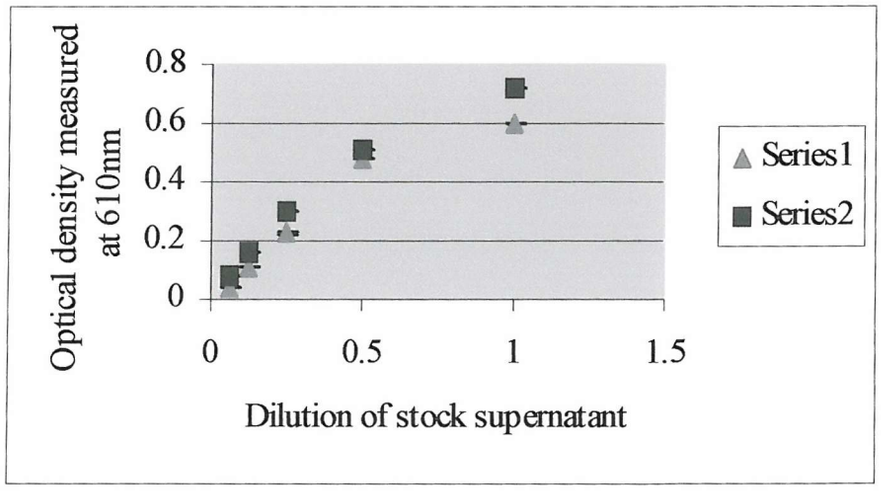
3.3 Choosing the technique for homogenisation.

Samples homogenised by both techniques were analysed in triplicate for the amount of total protein extracted. Both techniques generated homogenates that diluted in a similar fashion to the protein assay (see figure 3.4). Using the equation below, the coefficient of variation was determined for each technique, for samples of concentration "X" measured four times.

$$\text{The coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100 = X\%$$

The coefficient of variation for the "dismembranator" was 7% and for the "blender" was 9%. However the "blender" was easier to use, and extracted more protein from the tissue compared with the tissue dismembranator (see figure 3.4). Both techniques diluted similarly to the albumin standard. The "blender" system was chosen to homogenise all tissue samples from the study population.

Figure 3.4 ***Comparison of total protein extracted by "blender" and dismembranator" techniques.*** ***Series 1: tissue dismembranator. / Series 2: "blender" system***

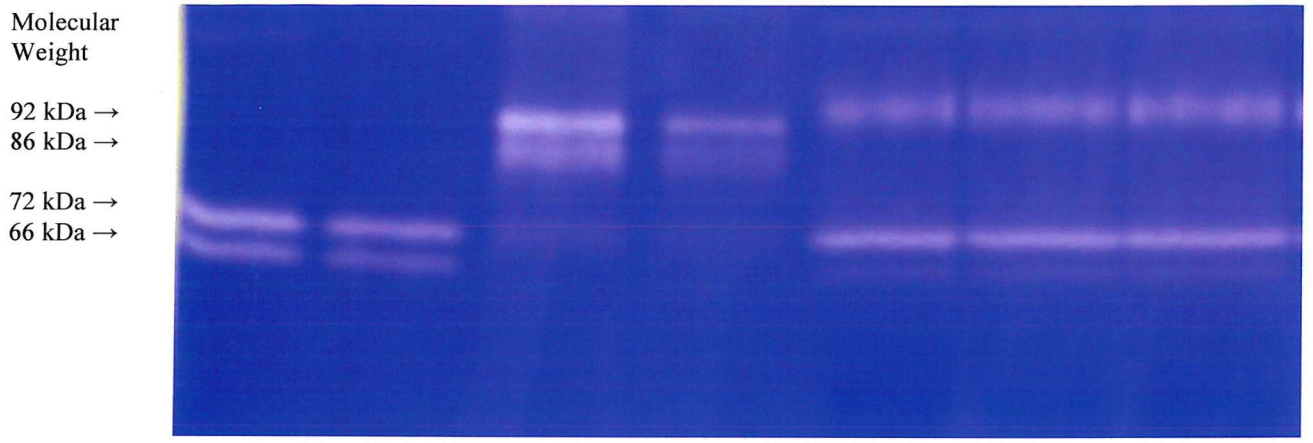


3.4 Zymography

Zymography was used to demonstrate and semi-quantify the expression of matrix metalloproteinase-2 and 9 (Bullen et al, 1995). This technique was chosen as it was well established and the same technique adopted by Jackson et al, (1996). The process was carried out by initially separating proteins within the sample by their molecular weight using gel electrophoresis. The substrate within the gels is the component gelatin. As MMP-2 and 9 are members of the "gelatinases" the migrated enzymes degrade and dissolve the incorporated gelatin. The gelatin is then stained blue and white bands appear where the gelatin has been degraded by MMP's-2 and 9 and hence doesn't take up the stain. As can be seen in figure 3.5, two bands can be identified at 72 kDa and 66 kDa in the first two columns and 92 kDa and 86 kDa in the next two columns. These correspond to the pro and active forms of MMP-2 and MMP-9 respectively. In vivo, only the active forms of MMP's can degrade gelatin. However, the addition of SDS to the samples causes unraveling of the pro-enzyme and reveals the active site of the enzyme, allowing it to degrade the gelatin.

Semi-quantification of enzyme expression was made using transmittance densitometry of the bands using a Sharp JX-330P flatbed scanner (Sharp Cooperation, Japan) and a computerised gel analysis package by Phoretix (Newcastle, U.K.)

Figure 3.5 ***Zymogram showing the separation of MMP-2 and MMP-9 by gel electrophoresis. Gelatin has stained blue and white bands illustrate where the gelatin has been digested. Pro-MMP-2=72 kDa; Active MMP-2=66 kDa; Pro-MMP-9=92 kDa; Active MMP-9=86 kDa.***



3.5 Protocol for zymography

A 0.75mm thick, 8% resolving gel was made between 2 glass plates by mixing the constituents below, and allowed to set for approximately 30 minutes.

	8% Resolving Gel
40% Acrylamide/Bis	2000 µl
1.5M Tris pH 8.8	2500 µl
Distilled water	1500 µl
Gelatin 2mg/ml	3900 µl
10% SDS	100 µl
TEMED	6 µl
10% APS	65 µl

Thereafter a 10-toothed comb was inserted above the resolving gel, and a 4% stacker gel solution (outlined below) was mixed and added between the teeth of the comb. The stacker gel was allowed to set for 90 minutes (see figure 3.6).

	4% Stacker Gel
40% Acrylamide/Bis	500 µl
0.5M Tris pH 6.8	1260 µl
Distilled water	3180 µl
10% SDS	50 µl
TEMED	5 µl
10% APS	25 µl

After the stacker gel solution had set the comb was removed, leaving wells in the top of the stacker gel into which our samples could be loaded. 15 µl of sample was added to 5 µl of sample buffer (0.25M tris HCl, 10% SDS, 5% sucrose, 0.1% bromophenol, pH to 6.8). The sample and sample buffer were mixed in a vortex mixer and then 15 µl of this mixture was loaded into the well. Initially 7 µl of a Kaleidoscope marker (Biorad, Hemel Hempstead, U.K.) was run alongside samples to demonstrate molecular weights. Once all samples were loaded, gels were run in electrophoresis buffer (6g Tris, 28.8g glycine, 2g SDS, made upto 2L with distilled water) at 80 volts for 90 minutes, (see figure 3.7) (Biorad powerpac 300, Biorad, Hemel Hempstead, U.K.).

Once electrophoresis was complete, the SDS was displaced by thoroughly washing the gels with 2.5% Triton-X100 thereby re-naturing the enzymes. The gels were then incubated for 18 hours in a buffer suitable to allow proteolysis of the gelatin by the migrated MMP enzymes (0.05M Tris, 5mM $CaCl_2.2H_20$, 0.02% sodium azide, pH to 8) The SDS in the sample buffer and gels allows the proteolytic sites on the pro-MMP enzymes to become activated and hence degrade the gelatin.

Figure 3.6 ***Preparation of gel between two glass plates. The white comb can be seen at the top of the gel which leaves wells for the samples to be loaded.***

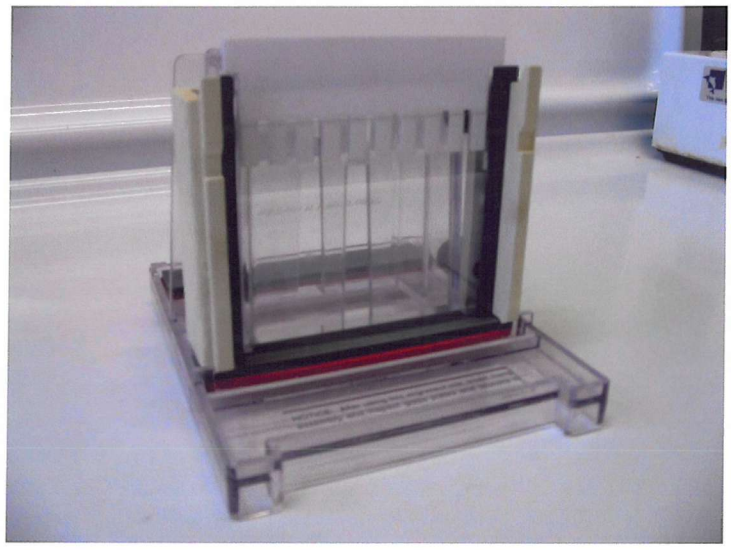
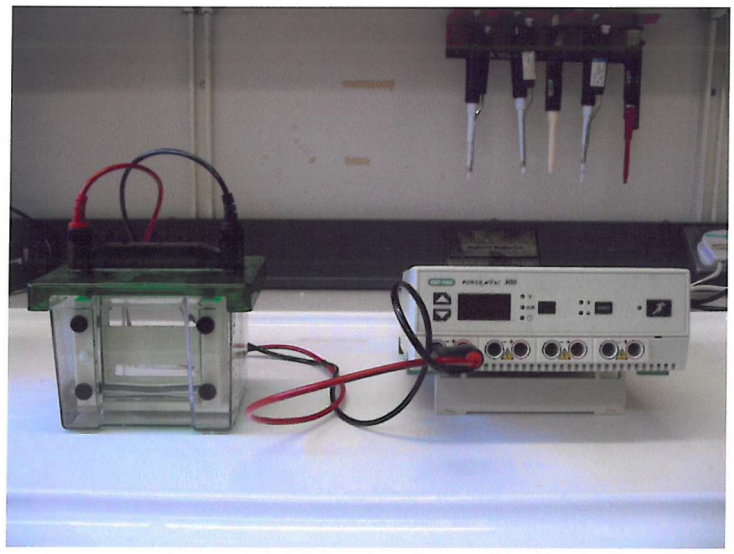


Figure 3.7 ***Electrophoresis of gels. Electrophoresis tank connected to Bioorad powerpac-300.***



After 18 hours of incubation, gels were washed thoroughly and then stained with Coomassie blue stain for 1 hour (50% methanol, 17% acetic acid, 2% Coomassie reagent R280 [Pierce cat No: 2320, Illinois, U.S.A.]) Gels were then immersed in a destain for 20 minutes (150ml 30% methanol, 35ml 10% acetic acid, 300ml distilled water). The zones where proteolysis of the gelatin had occurred were visualised as white bands where the blue staining of the gelatin had not occurred.

Relative enzyme quantitation was determined by transmittance densitometry of the gel with a flatbed optical scanner (Sharp JX330P at 300 dpi, Sharp Cooperation, Japan) and computerised gel analysis package (Phoretix, Newcastle, U.K.).

3.6 Establishing a standard curve for zymograms

A gelatinase zymography standard mixture of both human MMP-2 and human MMP-9 were acquired from Chemicon International Inc.(Harrow, U.K. Catolog No. CC073. Lot No. 18090872). The concentration of the standards was 0.1mg of protein per ml of buffer (5mM Tris - 0.1mM $CaCl_2$ - 0.005% Brij 35, pH 7.5). The company was contacted to inquire what the concentration of each enzyme was, as the total purified protein concentration was 0.1mg/ml. Unfortunately Chemicon were unable to provide an accurate concentration. It was therefore decided to express enzyme expression as arbitrary units (au) as measured by densitometry of gels. This is similar to the methodology used by Jackson et al (1996).

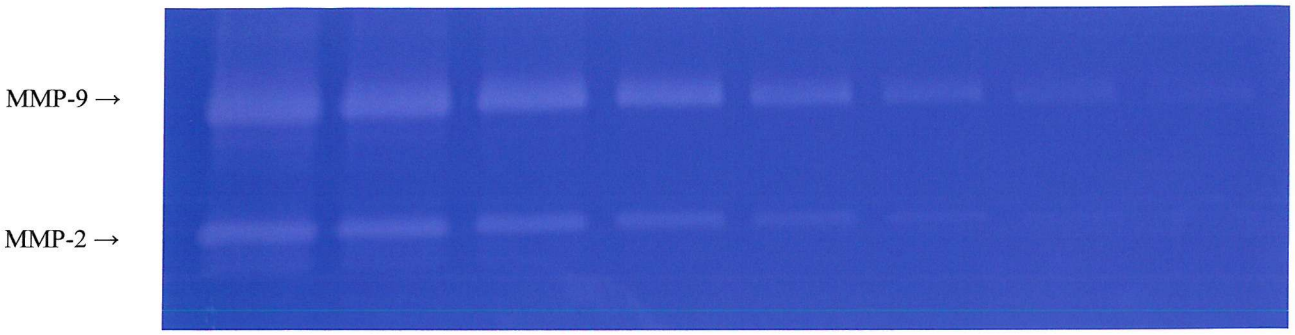
The MMP-2 / 9 standard was diluted with Phosphate Buffered Saline (PBS), making a "working standard" of 1 μg standard / ml PBS. Serial dilutions of this working standard were then made: 1 in 1, 1 in 2, 1 in 4. 1 in 8, 1 in 16, 1 in 32, 1 in 64,and 1 in 128. These dilutions of the zymography working standard were then analysed using substrate electrophoresis as described earlier. The intensity of each band was determined using flatbed densitometry (Sharp JX 330P) and a gel analysis package by Phoretix. By measuring the intensity of the bands for each dilution of the standard a standard curve was determined for each, as shown in figure 3.8.

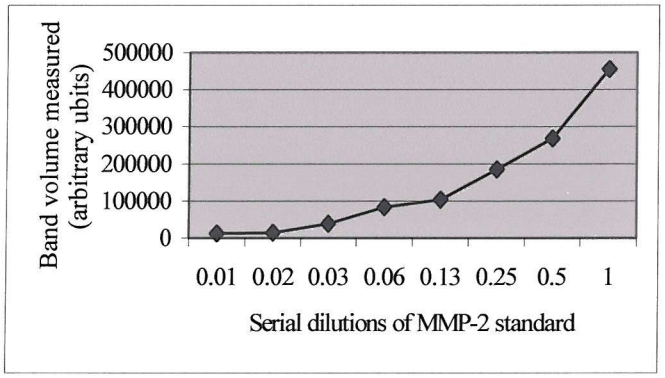
Dilutions of standard were chosen to lie in the linear portion of the standard curves for MMP-2 and MMP-9, so that only two dilutions of the standard were required for

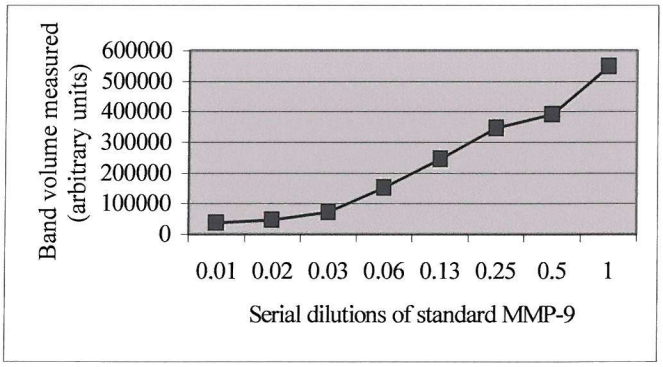
subsequent gels upon which the unknown samples were being analysed. By including two standards on each individual gel, the introduction of error that may occur between different gels and different runs was excluded. No more than 2 standards could be placed per gel because of limitations in the number of available wells (see figure 3.8).

Serial dilutions of tissue "stock supernatant" were made to determine an approximate working dilution for tissue samples. It was found that samples diluted 1:1-1:4 easily demonstrated relative enzyme concentrations that fell within the most linear portion of the MMP-2 and MMP-9 standard curves.

Figure 3.8 ***Zymogram of serial dilutions of human MMP-2 and human MMP-9 standards. Below are the resulting standard curves for each enzyme.***







3.7 Reproducibility of zymography

Transmittance densitometry of gels is a semi-quantitative technique. The variation of the zymography was assessed by analyzing the reproducibility. Supernatant of "stock" sample was diluted 1 in 2 with Dulbecco's PBS and loaded into 5 wells on one gel. The coefficient of variation was calculated using the equation:

$$\text{The coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100 = X\%$$

The coefficient of variation for the zymography process was calculated at 10%. It was shown that within the same gel reproducibility is extremely good, however, the variation between measurements of the same sample increased when analysed on different gels within the same run due to differences in the staining and de-staining of the gels (Coefficient of variation varies = 20-30% between gels). Therefore, samples from matched patients were analysed on the same zymogram, and each gel had two reference standards.

3.8 Loading of samples and calculation of enzyme concentrations within the study population.

The total protein extracted in each of the samples was measured as described in section 3.2 of the Methodology (diluting all samples x10 to measure the protein content to ensure that readings were on the linear portion of the standard curve). Protein concentrations within each sample were then calculated and compared, then approximately the same concentration of protein was loaded into each well of the zymograms. This meant that even by cursory inspection one could visualize relative proportions of enzyme from one sample to another. An explanation of the preparation of samples is shown in the following example:

2 patients X and Y are matched for age parity and hormonal status.

	[protein] (mg/ml)	X10 (mg/ml)	Vol.sample + (ml)	Vol PBS (ml)	[protein] loaded in well (mg/ml)
Patient X: ligament:	0.210	2.1	60	0	**2.1**
Patient X: vaginal skin:	0.230	2.3	60	0	**2.3**
Patient Y: ligament:	0.448	4.48	30	30	**2.24**
Patient Y; vaginal skin:	0.405	4.05	30	30	**2.03**

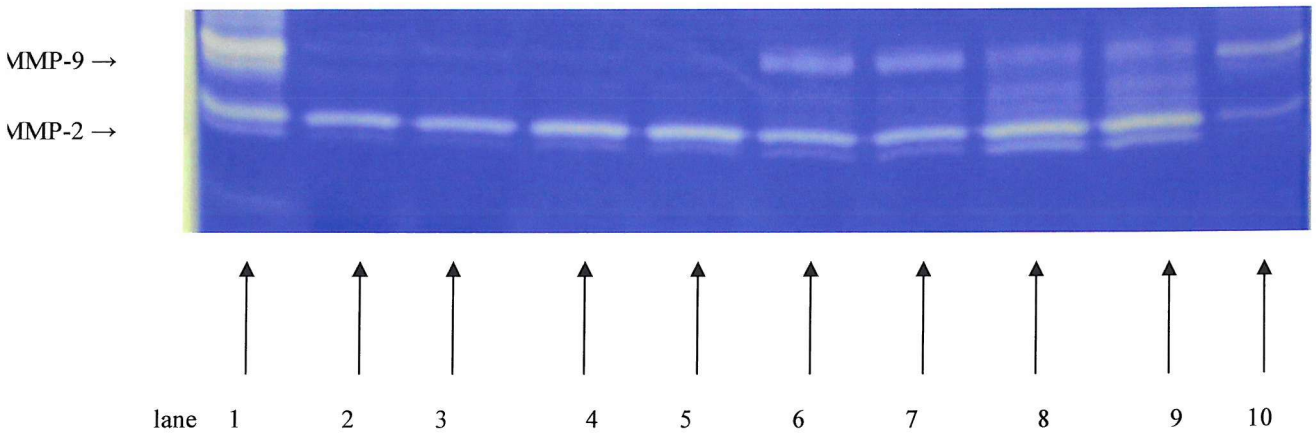
After zymograms had been analysed all enzyme concentrations were divided by the exact protein concentration loaded into each well and expressed as relative enzyme concentration (arbitary units) per mg of protein.

All zymograms were then run to the following configuration:

Lane 1	Human MMP-2 and MMP-9 standard (diluted 1 in 4)
Lane 2	Patient "X": ligament
Lane 3	Patient "X": ligament
Lane 4	Patient "X": vaginal skin
Lane 5	Patient "X": vaginal skin
Lane 6	Patient "Y": ligament
Lane 7	Patient "Y": ligament
Lane 8	Patient "Y": vaginal skin
Lane 9	Patient "Y": vaginal skin
Lane 10	Human MMP-2 and MMP-9 standard (diluted 1 in 8)

This configuration can be seen in figure 3.9.

Figure 3.9 Zymogram of 2 patients matched for age and parity.



This figure demonstrates the results for two patients "X" and "Y". On cursory inspection one can see that the expression of both MMP-2 and MMP-9 were increased in both the uterosacral ligaments (lanes 6+7) and vaginal skin (lanes 8 +9) of patient "Y" compared with the ligaments (lanes 2+3) and skin (lanes 4+5) of patient "X".

3.9 Enzyme Linked Immunosorbent Assay (ELISA) for TIMP-2

As outlined in section 2 of the introduction, MMP activity is determined by 1) the amount of pro-enzyme produced, 2) the amount of active enzyme released and 3) by the action of local tissue inhibitors (TIMP's). TIMP-2 is involved in the regulation of MMP-2 and to some extent MMP-9. The amount of TIMP-2 in samples was measured in order to allow for any possible inhibition of MMP-2 activity. TIMP-2 expression was measured using an ELISA system for human TIMP-2 supplied by Amersham Pharmacia: Biotrak Cellular Communication Assays: code RPN 2618 (Bucks, UK).

Working standards of 8ng/ml, 16ng/ml, 32ng/ml, 64ng/ml, 128ng/ml and 256ng/ml were prepared from a stock standard of human TIMP-2, (256ng/ml TIMP-2 in 0.03M phosphate buffer pH 7, containing 0.1M sodium chloride, 0.3%(w/v) bovine serum albumin and 0.01M EDTA in 1ml distilled water: provided by Amersham), and diluted in assay buffer to a volume of 110µl.

110µl of assay buffer (10ml phosphate buffer concentrate diluted to 100mls with distilled water, to give 0.03M phosphate buffer pH 7, containing 0.1M sodium chloride, 0.3%(w/v) bovine serum albumin and 0.01M EDTA: provided by Amersham) was used for the zero standard. 110 µl of the unknown samples were loaded into separately labeled tubes. 110µl of peroxidase conjugate (lyophilised anti-TIMP-2 horseradish peroxidase reconstituted in 12ml distilled water, in 0.03M phosphate buffer pH 7, containing 0.1M

sodium chloride, 0.3%(w/v) bovine serum albumin and 0.01M EDTA: provided by Amersham) were added to each tube and vortex mixed.

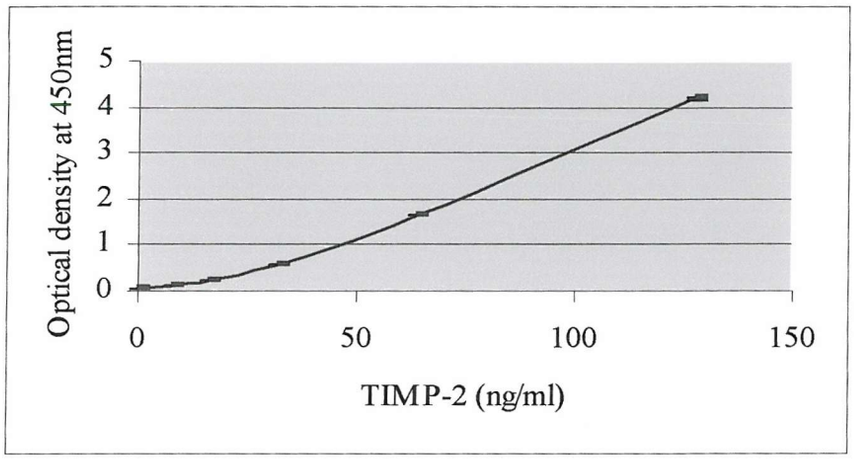
100µl of the mixture of standards or samples and peroxidase conjugate were loaded in duplicate into the wells of the microtitre plate coated with anti-TIMP-2. The wells of the microtitre plate were covered with the lid provided by Amersham and incubated at room temperature for exactly 2 hours. After incubation the wells were aspirated and washed 4 times with wash buffer (12.5ml phosphate buffer diluted to 500ml with distilled water to give a 0.01M phosphate buffer pH 7.5 containing 0.05M sodium chloride and 0.05% Tween^{TM}20).

The plate was blotted on tissue paper and wells then immediately loaded with 100µl of TMB substrate (22ml of 3,3',5,5'-tetramethlybenzidine (TMB)/hydrogen peroxide in 20%(v/v) dimethylformamide: provided by Amersham). The plate was covered and left at room temperature for exactly 30 minutes. After 30 minutes the reaction was stopped by the addition of 100µl 1M sulphuric acid to all wells and the plate was read at 450nm on a 96 well plate reader. Optical densities for each standard were calculated to generate a standard curve and TIMP-2 expression was then calculated for each of the unknown samples.

The standard curve for TIMP-2 is linear (see figure 3.10). A test run using 12 wells of the microtitre plate was initially performed to see where along the standard curve the concentration of TIMP-2 from the unknown samples would lie. Samples for the test run,

were taken from the same stock supernatant as used in the zymography. The preliminary test showed that undiluted stock supernatant lay in the linear portion of the standard curve. A final run of all samples was performed when all samples had been harvested and homogenised and measured in the same assay to reduce any assay variation.

Figure 3.10 Standard curve for TIMP-2 ELISA: (mean +/- sem)



Concentration of TIMP-2 for each sample was calculated from the standard curve and then these concentrations were divided by the protein concentration of each individual sample. Therefore TIMP-2 concentration is expressed as ng TIMP-2 / mg total protein.

3.10 Reproducibility of TIMP-2 Assay

Figures for coefficient of variation are quoted from the data supplied from Amersham. The data was derived from measuring duplicate controls within the assay and is outlined below.

Control	**Mean +/- SD**	**% CV**	**N**
A	16.3 +/- 0.88	5.4	12
B	31.6 +/- 1.10	3.5	12
C	50.9 +/- 1.36	2.8	12

3.11 Hydroxyproline Assay

As explained in the introduction, hydroxyproline is one of the main amino acids used in forming collagen. In fact hydroxyproline accounts for 14% of the dry weight of collagen. It has now become an established technique to measure hydroxyproline concentration in tissue to gain an idea of total collagen content (Stegeman et al, 1967; Keane et al, 1997; Jackson et al, 1996; Sayer et al; 1990). The method involves comparing the concentration of hydroxyproline extracted from hydrolysed tissue samples, with a standard curve made from serial dilutions of a known concentration of hydroxyproline. By multiplying the hydroxyproline content by 7.14 one can gain a reliable estimate of collagen content. The protocol for the hydroxyproline assay is described below.

STEP 1:

Tissue samples were blot dried and weighed. Samples were added to 2ml of 6N HCl, at 105°C and left until hydrolysed to dryness. This usually took 12-24 hours. Hydrolysed samples were diluted with distilled water (see 3.12 for dilution).

Standard concentrations of hydroxyproline were prepared as described below:

5mM stock = 65.565mg of hydroxyproline in 100ml 6N HCl.

1mM stock = 10ml (5mM) + 40ml 6N HCl.

Hydroxyproline standard:	**mL stock**	**mL 6N HCl**
1.5mM	1.5 (5mM)	3.5
1mM	1 (5mM)	4
0.8mM	4 (1mM)	1
0.6mM	3 (1mM)	2
0.4mM	2 (1mM)	3
0.2mM	1 (1mM)	4

STEP 2:

Once prepared, 200 µl of each standard was added to 200 µl of distilled water

All aliquots of standards and hydrolysed samples were taken to dryness at 105 °C. 1ml of citrate buffer (14.7g tri-sodium citrate, 9.2g citric acid, 500mL distilled water, mixed with 5 volumes of propan-2-ol [eg. 30ml stock + 150ml propan-2-ol] was added to dried aliquots and shaken on a multi-vortexer for 30 minutes

STEP 3:

250 µl of chloramine T reagent (40ml of buffer: [28.44g sodium citrate, 18.82g trisodium citrate, 2.52g citric acid, 1981ml distilled water, 309ml propan-2-ol] added to 0.7g chloramine-T + 10ml distilled water) was added to each tube and incubated for exactly 15 minutes.

STEP 4:

After 15 minutes samples were immediately placed in an ice bath to arrest the reaction. Then 2.0 ml of Ehrlich's reagent (6.67g p-dimethyaminobenzaldehyde, 11ml concentrated HCl, 89ml propan-2-ol) was added to each tube. Tubes were then capped and left overnight at room temperature

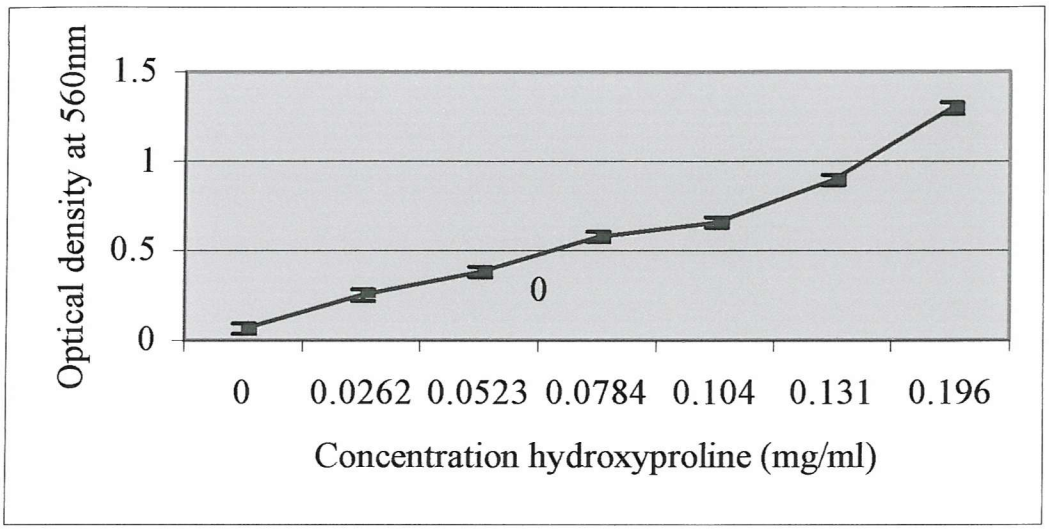
STEP 5:

The next morning tubes were mixed on the multi-vortexer and then left for 1 hour
The optical density of the samples and standards were read in a Dynex Technologies MRX 96 well plate reader at 560nm. Hydroxyproline concentration within samples was then calculated from the standard curve.

3.12 Establishing the "working concentration" for hydroxyproline assay

Numerous preliminary assays were performed to familiarise with the assay. During these assays the working dilution of hydrolysed samples was determined. Samples of dried stock tissue were hydrolysed as explained in step 1 of the protocol, and then diluted at the dilutions of 1 in 2 (ie 200 µl sample + 200 µl of distilled water), 1 in 10 and 1 in 100. Steps 2-5 were then completed and hydroxyproline content measured against standards. On this basis it was shown that samples needed to be diluted 1 in 10 to lie within on the linear portion of the standard curve (see figure 3.11).

Figure 3.11 Standard curve for hydroxyproline assay (mean +/- sem)



3.13 Reproducibility of the hydroxyproline assay

The hydroxyproline assay was already established within another laboratory in the University. Reproducibility studies had previously been performed. However, inter and intra – assay variability studies were performed. Intra-assay coefficient of variation was calculated at 7%, but the inter-assay coefficient of variation was 25%. Hence, all samples were run in the same assay once all samples had been collected. To ensure results were as accurate as possible samples were hydrolysed in duplicate and each aliquot was run in duplicate on the plate reader. Hence for each sample, four results were obtained and the mean reading for each sample taken.

METHODOLOGY

Chapter 4:

Immunocytochemistry

Contents to Chapter 4:

Chapter 4: Immunocytochemistry

4.1 Fixation and preparation of tissue sections

Immunocytochemistry is a technique used to visualise the distribution and proportion of expression of specific cell antigens. This is achieved by labeling an antibody specific to the antigen in question, allowing visualisation of tissue containing that antigen when the labeled antibody is bound to it.

As the relationship between oestrogen receptor expression and smooth muscle phenotype was of interest, only samples of uterosacral ligament were examined for histology. The uterosacral ligament samples were bisected. One half was immersed in liquid nitrogen and analysed for collagen metabolism. The other half was placed in 10% buffered formalin fixative for at least 24 hours. The purpose of fixation is to preserve all components of a tissue sample in their true situation and protect the tissue from osmotic damage (swelling or shrinkage). Formalin is a cross-linking fixative, and fixes the tissue by forming hydroxy-methylene bridges between the reactive end groups of adjacent protein chains. Fixed proteins can only retain their antigenicity if the cross-linking does not affect the amino acid sequences that bind to the antibody used in the immunocytochemistry. Once fixed for 24 hours, samples were processed and embedded in paraffin wax. They can then be stored indefinitely at room temperature and still retain their antigenicity.

Paraffin sections were cut to a width of 5μm and floated on warm water. Every attempt was made to cut serial sections. Sections were floated onto glass slides coated with aminopropyltriethoxysilane (APES) which provide an adhesive for the tissue to stick to the glass in an attempt to prevent tissue migration from the slide during antigen retrieval (see later). Slides were left to dry overnight in an incubator set at 37°C and then stored at room temperature.

4.2 Blocking of endogenous peroxidase enzyme

The immunocytochemical reaction involves labeled antibodies binding to specific antigens. Visualisation of the immunocytochemical reaction occurs through enzyme markers linked to the antibody. These enzymes produce a coloured end-product on the addition of a substrate (usually brown or red) demonstrating the antigen's presence. Therefore any endogenous enzyme of the same type as the label must be inhibited (blocked) so that it cannot react with the substrate and be confused with the applied label. In this study peroxidase was used as the enzyme, and hence any endogenous peroxidase needed to be blocked. Slides were appropriately labeled in pencil, noting the sample number and the antibody they were to be stained with. They were then de-paraffinised in xylene and rehydrated through graduated alcohols to 70% then Tris buffered saline (ph 7.6). Sections were then immersed in 0.3% hydrogen peroxide in methanol for 10 minutes. This step is to block endogenous peroxidase activity. Peroxidase is present in peroxisomes and macrophages. A related enzyme *catalase* is present within red blood cells. The addition of hydrogen peroxide and methanol, suppresses the activity of these

enzymes and is necessary before the application of any peroxidase linked reagent used later in the assay.

4.3 Antigen retrieval

After blocking endogenous peroxidase activity, the next step is to retrieve antigens masked by formalin fixation. During formalin fixation, the formation of hydroxy-methylene bridges between amino acid chains leads to a reduction in the antigenicity of certain proteins. In many instances the immunoreactivity can be restored without compromising the structure of the tissues. Numerous techniques can be used to break the cross-links to restore antigenicity before immunostaining. These include enzymatic degradation of the cross-links with protease enzyme and heat mediated cleavage of the bonds. Heating deparaffinised sections in citrate buffer, either in a microwave or pressure cooker leads to an increase in antigenicty of certain antigens. Morgan et al (1994), suggested that heating samples in citrate provides the energy not only to rupture the hydroxyl bonds formed by the fixative with the protein, freeing some antigens, but also releases tissue bound calcium ions which contribute to tighter bonds with the fixative. The antigenic sites for desmin, vimentin and α – smooth muscle actin were all exposed by microwaving sections in 0.1M citrate buffer on medium power (650Watts) for 25 minutes. The antigenic site for the oestrogen receptor was exposed by boiling the sections in 0.1M citrate buffer in a pressure cooker at 13lb pressure for 2 minutes. After this process sections were washed in tris buffered saline ready for addition of the primary antibody.

4.4 Visualisation of the antigen

In order for an immunocytochemical reaction to be seen in the microscope, a component of the reaction must carry a label. There are numerous layers, labels and techniques to magnify the reaction available. A three layer technique was used in this study, as described below. The first layer is the primary antibody (usually IgG) raised to the antigen to be localized. Negative controls had a primary layer of non specific immunoglobulin of the same class and subtype from the same species as the primary antibody. The specific antibodies and their dilutions are outlined in table 4.I and table 4.II.

Table 4.I: List of antibodies and positive / negative controls

Antigen	**Primary Antibody**	**Isotype**	**Positive control Tissue**	**Negative Control**	**Secondary Antibody**
Oestrogen Receptor	Monoclonal anti -estrogen receptor Clone: **ID5** (DAKO)	Mouse IgG1, kappa	Breast tissue from women with carcinoma of the breast	Non specifc mouse IgG1, kappa (DAKO)	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)
Vimentin	Monoclonal anti-vimentin Clone: **Vim 3B4** (DAKO)	Mouse IgG2a, kappa	Colon from patients with Crohn's disease	Non specifc mouse IgG2a, kappa (DAKO)	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)
Desmin	Monoclonal anti-desmin (in house)	Mouse IgG1, kappa	Colon from patients with Crohn's disease	Non specifc mouse IgG1, kappa (DAKO)	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)
α smooth muscle actin	Monoclonal anti-α SMA (Sigma)	Mouse 1gG2a, kappa	Colon from patients with Crohn's disease	Non specifc mouse IgG2a, kappa (DAKO)	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)

Table 4.II: List of dilutions used for primary and secondary antibodies

Antigen	Primary Antibody	Dilution of primary antibody in TBS	Method of antigen retrieval	Secondary Antibody	Dilution of secondary antibody in TBS
Oestrogen Receptor	Monoclonal anti - estrogen receptor Clone: ID5 (DAKO)	1:400	Pressure cooker, 0.1M citrate buffer	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)	1:200
Vimentin	Monoclonal anti-vimentin Clone: Vim 3B4 (DAKO)	1:1000	Microwave, 0.1M citrate buffer	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)	1:200
Desmin	Monoclonal anti-desmin (in house)	1:1000	Microwave, 0.1M citrate buffer	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)	1:200
α smooth muscle actin	Monoclonal anti-α SMA (Sigma)	1:40,0000	Microwave, 0.1M citrate buffer	Biotinylated $F(ab')_2$ Rabbit anti-mouse Ig (DAKO)	1:200

Suppliers:

DAKO: High Wycombe, Bucks, U.K.

SIGMA: Poole, Dorset, U.K.

The primary antibody was diluted in TBS at the dilutions specified in table 4.II. Four drops were applied to each slide, enough to cover the tissue sections, and left to incubate at room temperature for 30 minutes. The sections were then washed three times with TBS and then shaken dry. The primary antibody is in itself not labeled, but is detected by a secondary antibody raised to the immunoglobulin of the species that donated the primary antibody. Thus, if the primary antibody is rabbit IgG anti-antigen, then the second antibody may be goat (or swine or sheep) anti-rabbit IgG. In this study all primary antibodies were derived from mouse so rabbit anti-mouse IgG was used as the secondary antibody. The secondary antibody was diluted in TBS as specified in table 4.II and four drops of the secondary antibody were applied over each section and allowed to incubate at room temperature for 30 minutes. The secondary antibody is "biotinylated", in other words is conjugated with biotin molecules attached to the Fc fragment. The third layer is the addition of avidin-biotin complexes.

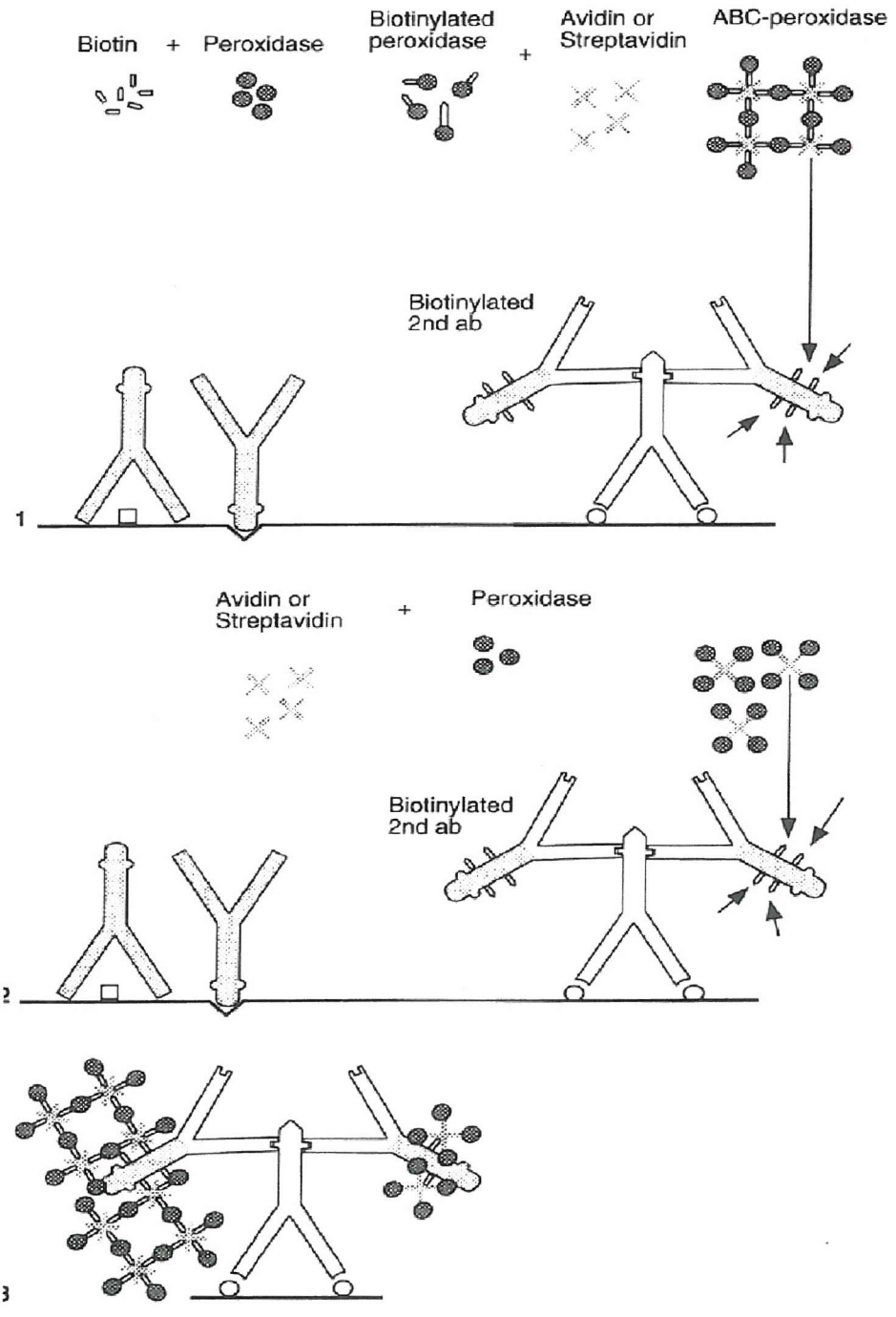
Avidin is a large glycoprotein extracted from egg white (albumin). It has four high affinity binding sites per molecule, for a low molecular weight vitamin called biotin, but can also be bound to other macromolecules (such as an enzyme) via other binding sites. Biotin is a vitamin extracted from egg yolk. Each biotin molecule has one binding site for avidin and can be attached through other sites to the secondary antibody or other macromolecules such as an enzyme or label. In this study the secondary antibody was biotinylated so the third layer which was a complex of avidin and biotin that has been labeled with peroxidase enzyme could bind to the secondary antibody. The avidin-biotin complex comes in a kit form from DAKO, (Code No. K 0377, Lot 101, High Wycombe,

Herts., U.K.). The package consists of a bottle of reagent A (Streptavidin) and a bottle of reagent "B" (biotinylated horseradish peroxidase). For every 50 slides, 1 drop of reagent "A" was mixed with 1 drop of reagent "B" in 5 mls of tris buffered saline. Four drops were then applied to each slide and allowed to complex for 30 minutes. Slides were then washed in TBS three times and shaken dry. The avidin and labeled biotin were allowed to complex for 30 minutes before being applied, resulting in large highly labeled complexes. This technique improves the sensitivity of the immunoreaction allowing only a few antigens to be easily visualized (Coons et al, 1986), (see figure 4.1).

Finally the substrate for the peroxidase was applied. In this study diaminobenzidine (DAB) was applied to the slides. The end product was a dark brown stain localized to the antigen location. Liquid DAB is used in a kit form supplied by DAKO (High Wycombe, UK. Cat No. HK 153-5K, HK 127-5K. The kit comprises of a bottle of substate buffer (HK 127-5K), a bottle of liquid DAB chromogen (HK 124-7K) and a bottle of hydrogen peroxide substrate (HK 126-7K). For every 50 slides, 0.5mls of substrate buffer, 4 drops of chromogen and 2 drops of hydrogen peroxide were added to 4.5mls of de-ionised water. Four drops of this mixture were then applied to each tissue section and left to incubate for 5 minutes. The brown staining could be visualized during this time. After 5 minutes slides were rinsed in TBS and washed in running tap water.

Sections were then counterstained with Mayers haematoxyllin for 1 minute then left in running tap water for 5 minutes. This allows the nuclei to turn blue making visualization easier. Finally the sections were dehydrated through graduated alcohols to xylene and mounted in DPX. They were stored at room temperature ready for analysis.

Figure 4.1 Three layer immunocytochemistry using the avidin-biotin complex (ABC) method



4.5 Analysis of oestrogen receptor concentration

The oestrogen receptor expression was quantified by counting positively stained nuclei, as was described by Bernstein et al, (1999). As explained in the introduction section, this method was adopted rather than ELISA which was used by Sundberg (1981) and Smith (1993) as it prevents contamination of samples from other surrounding tissues which may contain the oestrogen receptor. In addition immunohistochemistry allows localisation as well as quantification of the oestrogen receptor. Experiments demonstrated only uterosacral ligament smooth muscle cell nuclei stained for the oestrogen receptor as demonstrated in figure 5.2. There were no oestrogen receptors demonstrable in the nuclei of smooth muscle in blood vessels. Nuclei were counted manually using a graticule and hand counter. 1000 smooth muscle cell nuclei were counted and then the nuclei which were positively stained with the ID5 antidody were counted (see figure 5.3). Counting 1000 nuclei provides reproducible results in sections with both a high and low density of oestrogen receptor expression (Gray 1996). In this study the coefficient of variation for counting the number of positively stained smooth muscle cell nuclei per 1000 smooth muscle cell nuclei was 3.18%. Nuclei were only counted if they met certain criteria:

- they had to be longitudinal in sectioning
- they had to be within the confines of the graticule.

This prevented nuclei that weren't smooth muscle being counted or the double counting of nuclei in two adjacent areas. Results were expressed as percentage of positively stained smooth muscle cell nuclei per 1000 smooth muscle cell nuclei.

4.6 *Localisation and quantification of smooth muscle cell phenotypes*

The expression of vimentin, desmin and α-smooth muscle actin, were semi-quantified using the "Colourvision 1.7.6" computer image analysis software. For each slide stained, five areas of smooth muscle were outlined and the total area of smooth muscle calculated. Initially a "threshold" was set for the density of positively stained tissue compared with the background staining. This ensured that each measurement only measured areas of positive staining and excluded any non-specific background staining. As all the slides could not be assessed in one session a "standard" slide was used at the start of each session to set the "threshold". The aim of this was to improve the reproducibility of measurements. The coefficient of variation for the technique was calculated at 16%. The proportion of positively stained smooth muscle was measured in proportion to the total area of smooth muscle in five randomly chosen outlined areas to give a representative assessment (Gray 1996). The mean percentage of the total smooth muscle area that was positively stained was then calculated for each antigen.

RESULTS

Chapter 5:

Collagen metabolism

Contents to Chapter 5:

Chapter 5:

PART 1:

Collagen content and turnover in vaginal skin

5.1 Patient details

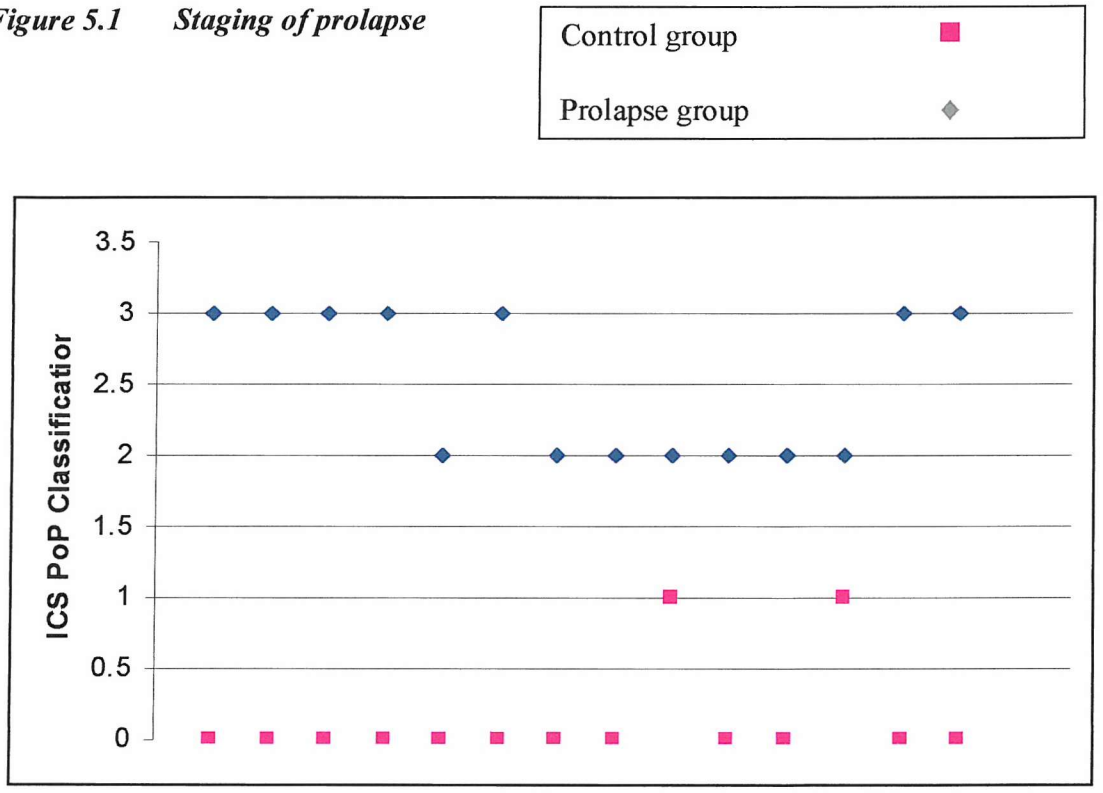
Of all women approached and recruited into the study in total 28 met the inclusion criteria and could be matched for age, race, parity and menopausal status. Women were further matched for phase of menstrual cycle or hormone replacement status where applicable. There were in total 16 postmenopausal women and 12 premenopausal women. The patient demographics are outlined below in table 5.I. Of the 28 matched patients 26 were caucasian and 2 were Asian. None of the women recruited for the study were Negro. There was no statistical significance between the control and study group in relation to age, parity or hormonal status.

Table 5.I Patient demographics

	Control group	**Prolapse group**
Age: mean *(range)*	60 *(36-84)*	57 *(38-87)*
Parity: mean *(range)*	2.5 *(0-6)*	3.1 *(0-7)*
Postmenopausal: no HRT	7	7
Postmenopausal: on HRT	1	1
Premenopausal: follicular	0	0
Premenopausal: secretory	6	6

All patients had a pelvic assessment preoperatively whilst awake, according to the International Continence Society pelvic organ prolapse classification, as outlined in Section 2.5. All measurements were made whilst the patient was performing the valsalva maneuver. The stage of prolapse in each patient is outlined below in Figure 5.1.

Figure 5.1 Staging of prolapse



5.2 Differences in: pro-MMP-2, active MMP-2. pro-MMP-9, TIMP-2 and hydroxyproline expression in vaginal tissue.

The control and prolapse groups were analysed as whole groups and then further sub-divided into premenopausal and postmenopausal states. Where data are paired a paired t-test was initially performed. However, as the populations were small and the data was sometimes skewed, the non-parametric Wilcoxon matched paired, sign ranked test was performed. When analyzing non-paired data (eg. comparing premenopausal and postmenopausal women in the same group) the Mann Whitney test was used as these cannot be paired. Although 28 women met the inclusion criteria and could be matched for age and parity, there was sufficient tissue available for complete analysis of all components in 26 women (13 pairs). In the remaining pair, analysis was done for hydroxyproline content and immunocytochemistry.

Pro-MMP-2 expression:

Zymography was performed on homogenised tissue extracted from patients in each group. Matched samples were loaded in duplicate onto the same gel adjacent to serial dilutions of a known concentration of standard MMP-2. All concentrations of the enzyme are expressed as arbitrary units (AU) per concentration of extracted protein (mg) as determined by transmittance densitometry (see section 3.5). There was no significant difference between the expression of pro-MMP-2 in the vaginal skin of premenopausal women controls compared with the postmenopausal controls (Mann Whitney test) (see

table 5.III). This was also true when comparing the premenopausal and postmenopausal women with prolapse.

There was a significant increase in pro-MMP-2 expression in the vaginal skin samples from women with prolapse compared with controls. This was true when the groups were analysed as whole and when divided into premenopausal and postmenopausal populations (see table 5.II and figure 5.2). Initially a paired t-test was performed as the data were paired and matched. However, as the data groups are small comparison of the data were finally tested using non-parametric Wilcoxon matched paired, sign ranked test which was thought to eliminate any bias that may be introduced if the populations were skewed. As can be seen in table 5.II there was no significant difference when the expression of pro-MMP-2 was compared in the vaginal tissue of premenopausal controls with that of postmenopausal controls. The same was true in women with prolapse. There was a significant increase in pro-MMP-2 expression in the vaginal tissue of women with prolapse compared with controls ($p<0.01$, $n=26$). When the groups were divided into their premenopausal and postmenopausal populations the p values were $p=0.0625$, $n=10$; $p<0.05$, $n=16$ respectively.

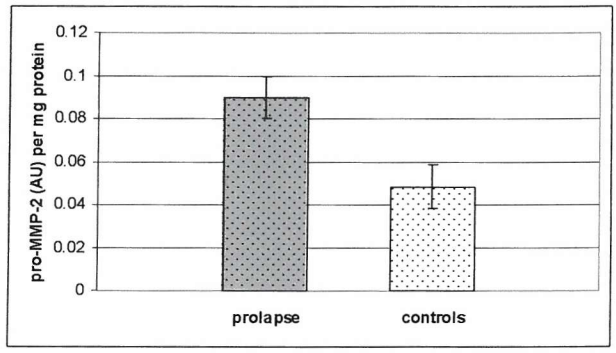
Table 5.II Comparisons of pro-MMP-2 expression in vaginal skin of premenopausal women and postmenopausal women

Comparison and numbers	Pro-MMP-2 expression mean and median	Statistical test	P value
Premenopausal controls (n=5) vs. postmenopausal controls (n=8)	0.038 0.032 vs. 0.0546 0.033	Mann Whitney	Not sig.
Premenopausal prolapse (n=5) vs. postmenopausal prolapse (n=8)	0.063 0.052 vs. 0.107 0.094	Mann Whitney	Not sig.
Controls (n=13) vs. prolapse (n=13)	0.048 0.031 vs. 0.090 0.084	Wilcoxon Paired t	0.0034 0.0094
Premenopausal controls (n=5) vs. premenopausal prolapse (n=5)	0.038 0.032 vs. 0.063 0.052	Wilcoxon Paired t	0.0625 0.039
Postmenopausal controls (n=8) vs. postmenopausal prolapse (n=8)	0.0546 0.033 vs. 0.107 0.094	Wilcoxon Paired t	0.0234 0.0412

Figure 5.2 ***Comparison of pro-MMP-2 expression in vaginal skin of women with prolapse compared with controls expressed as arbitrary units (AU) per mg protein. (mean +/-SEM)***

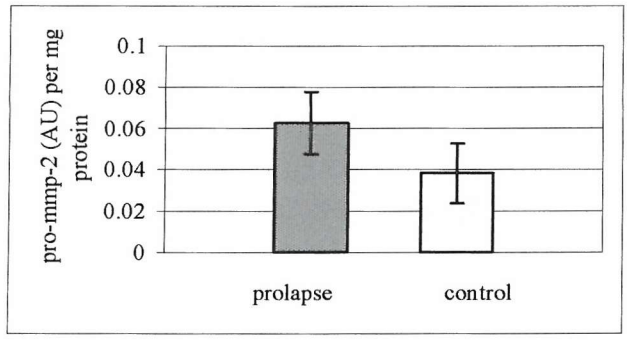
All prolapse vs. controls (n=26):

p<0.01 Wilcoxon
p<0.01 paired t test



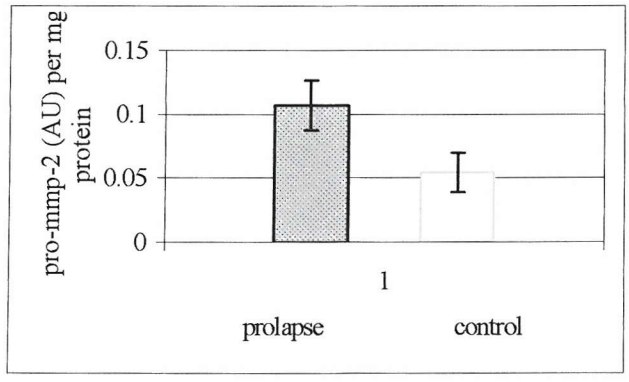
Premenopausal women (n=10):

p=0.0625 Wilcoxon
p<0.05 paired t-test



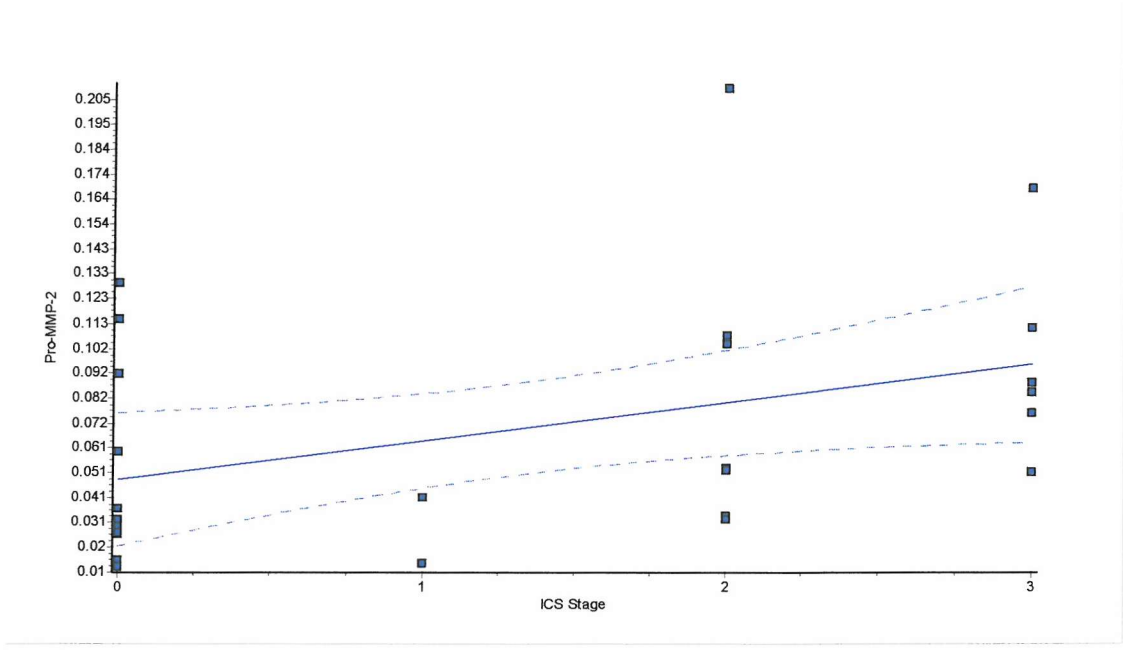
Postmenopausal women (n=16):

p<0.05 Wilcoxon
p<0.05 paired t test



In view of these interesting results the correlation between stage of prolapse and pro-MMP-2 expression was investigated. Figure 5.3 demonstrates there is a significant correlation between the stage of prolapse and the amount of pro-MMP-2 expressed in the vaginal tissue ($r=0.4$, $p<0.05$).

Figure 5.3 Correlation between the ICS POP classification: stage of prolapse and pro-MMP-2 expression (AU per mg protein), r=0.4, p<0.05



Active MMP-2 expression:

A second band was demonstrated on zymography at 66 kDa which corresponded to the active isoform of MMP-2. Transmittance densitometry was used to estimate the enzyme expression and is expressed as arbitrary units / mg total protein. Similar to pro-MMP-2, there was no significant difference between active MMP-2 expression in premenopausal controls and postmenopausal controls. A significant increase in the expression of active MMP-2 was found in skin samples from postmenopausal women with prolapse compared with premenopausal women with prolapse ($p<0.05$, Mann Whitney test), see table 5.III.

There was a small increase in the expression of active MMP-2 in vaginal skin samples from women with prolapse compared with controls (n=26), but this was not statistically significant. This was also true when the groups were divided into their premenopausal and postmenopausal populations (see table III).

Table 5.III Comparisons of active-MMP-2 expression in vaginal skin of premenopausal women and postmenopausal women

Comparison and numbers	Active-MMP-2 expression mean and median	Statistical test	P value
Premenopausal controls (n=5) vs. postmenopausal controls (n=8)	0.032 0.027 vs. 0.044 0.042	Mann Whitney	Not sig.
Premenopausal prolapse (n=5) vs. postmenopausal prolapse (n=8)	0.035 0.043 vs. 0.083 0.057	Mann Whitney	P<0.05
Controls (n=13) vs. prolapse (n=13)	0.039 0.039 vs. 0.064 0.052	Wilcoxon	Not sig.
Premenopausal controls (n=5) vs. premenopausal prolapse (n=5)	0.032 0.027 vs. 0.035 0.043	Wilcoxon	Not sig.
Postmenopausal controls (n=8) vs. postmenopausal prolapse (n=8)	0.044 0.042 vs. 0.083 0.057	Wilcoxon	Not sig.

Pro-MMP-9 expression:

A final band was demonstrated on zymography at 92 kDa. This corresponded to the enzyme pro MMP-9. Transmittance densitometry was used to estimate the enzyme expression and is expressed as arbitrary units / mg total protein. There was no significant difference between the expression of pro-MMP-9 in the vaginal skin of premenopausal women controls compared with the postmenopausal controls (Mann Whitney test) (see table 5.IV). This was also true when comparing the premenopausal and postmenopausal women with prolapse.

There was no significant difference in the expression of pro-MMP-9 in vaginal skin samples from women with prolapse compared with controls (n=26) or when comparing the groups after they had been divided into premenopausal and postmenopausal populations (see table 5.IV).

Table 5.IV Comparisons of pro-MMP-9 expression in vaginal skin of premenopausal women and postmenopausal women

Comparison and numbers	Pro-MMP-9 expression mean and median	Statistical test	P value
Premenopausal controls (n=5) vs. postmenopausal controls (n=8)	0.024 0.017 vs. 0.026 0.015	Mann Whitney	Not sig.
Premenopausal prolapse (n=5) vs. postmenopausal prolapse (n=8)	0.040 0.035 vs. 0.027 0.023	Mann Whitney	Not sig.
Controls (n=13) vs. prolapse (n=13)	0.025 0.035 vs. 0.032 0.026	Wilcoxon	Not sig.
Premenopausal controls (n=5) vs. premenopausal prolapse (n=5)	0.024 0.017 vs. 0.040 0.035	Wilcoxon	Not sig.
Postmenopausal controls (n=8) vs. postmenopausal prolapse (n=8)	0.026 0.015 vs. 0.027 0.023	Wilcoxon	Not sig.

TIMP-2 expression:

TIMP-2 activity within the homogenised tissue was measured using enzyme-linked immunofluorescence (ELISA) techniques. Samples for TIMP-2 analysis were taken from the same homogenate used for MMP analysis. Once again the amount of TIMP-2 (ng/ml) was measured in relation to the total protein (mg/ml) extracted. TIMP-2 concentration is expressed as ngTIMP-2 per mg total protein. There was no significant difference found between the expression of TIMP-2 in the vaginal skin of premenopausal women controls compared with the postmenopausal controls (Mann Whitney test) (see table 5.V). This was also true when comparing the premenopausal with postmenopausal women with prolapse. However, it is interesting to note the highest TIMP-2 expression was seen in the postmenopausal women with prolapse, (see table 5.V).

There was no significant difference in the expression of TIMP-2 in vaginal skin samples from women with prolapse compared with controls (n=26) or when groups were divided into the premenopausal and postmenopausal populations (see table 5.V).

Table 5.V Comparisons of TIMP-2 expression in vaginal skin of premenopausal women and postmenopausal women

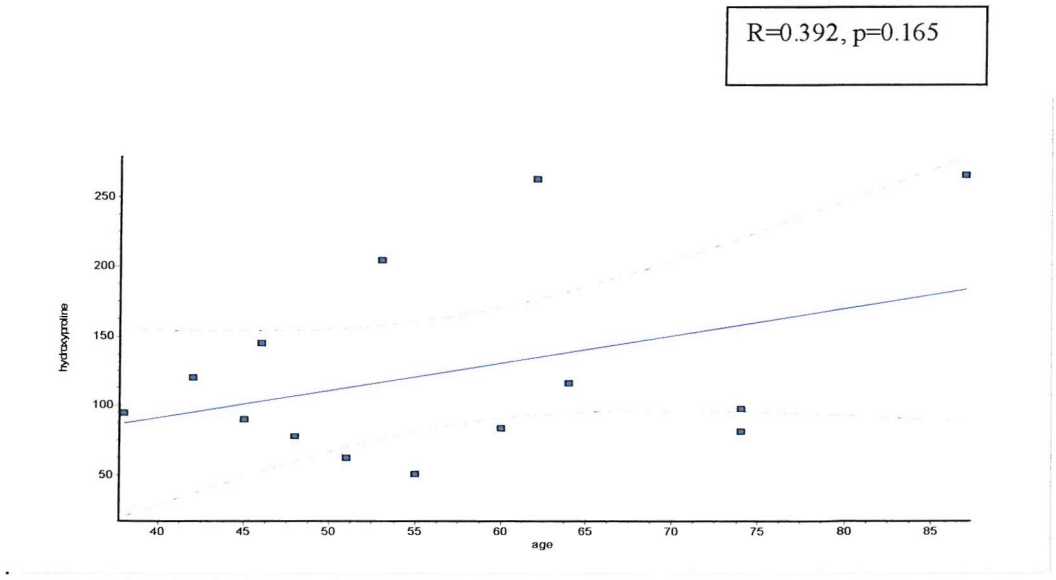
Comparison and numbers	TIMP-2 expression mean and median	Statistical test	P value
Premenopausal controls (n=5) vs. postmenopausal controls (n=8)	8.97 9.55 vs. 7.95 2.22	Mann Whitney	Not sig.
Premenopausal prolapse (n=5) vs. postmenopausal prolapse (n=8)	5.14 5.64 vs. 13.47 12.97	Mann Whitney	Not sig.
Controls (n=13) vs. prolapse (n=13)	8.34 6.46 vs. 10.27 7.28	Wilcoxon	Not sig.
Premenopausal controls (n=5) vs. premenopausal prolapse (n=5)	8.97 9.55 vs. 5.14 5.64	Wilcoxon	Not sig.
Postmenopausal controls (n=8) vs. postmenopausal prolapse (n=8)	7.95 2.22 vs. 13.47 12.97	Wilcoxon	Not sig.

Hydroxyproline content:

Relationship between hydroxyproline content and age

Because of previous work studying hydroxyproline content and age in other tissues (Brincat et al, 1983, 1987), it was of interest to see if the collagen content within normal vaginal tissue decreased with increasing age. This is often thought to be the case on inspection of an atrophic lower genital tract. The correlation between hydroxyproline content and age in women with no prolapse was therefore assessed. There was no significant change in the hydroxyproline content in the vaginal skin of controls with increasing age, (correlation coefficient r=0.392, p=0.165), see figure 5.4.

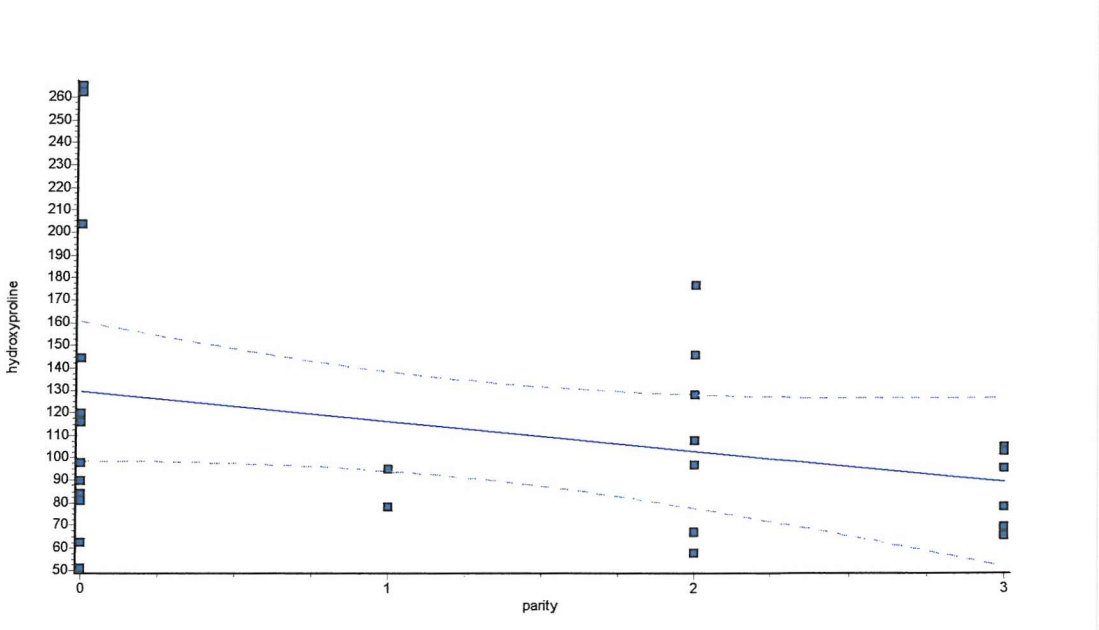
Figure 5.4 ***Correlation between hydroxyproline content (μmol/g dry weight tissue) and age in controls (years)***



Relationship between hydroxyproline content and parity

The relationship between hydroxyproline content and parity was examined in controls. There was no correlation between the parity of women and the hydroxyproline content of their vaginal tissue (r= - 0.3, p=0.12) as shown in figure 5.5.

Figure 5.5 Correlation between hydroxyproline content (μmol/g dry weight tissue) and parity in controls



The hydroxyproline content in dried samples were measured against standards (see section 3.11). The concentration of hydroxyproline is expressed as μmol/g blot dried tissue. There was no significant difference between the hydroxyproline content in the vaginal skin of premenopausal women controls compared with the postmenopausal controls (Mann Whitney test) (see table 5.VI). This was also true when comparing the premenopausal and postmenopausal women with prolapse. There was also no significant difference between the hydroxyproline content of vaginal biopsies from women with prolapse and that of controls (n=28), or when the groups were divided into premenopausal and postmenopausal populations as tested by the Wilcoxon Matched Paired Sign Ranked test.

Table 5.VI Comparisons of hydroxyproline content of vaginal skin in premenopausal women and postmenopausal women

Comparison and numbers	Hydroxyproline content mean and median	Statistical test	P value
Premenopausal controls (n=5) vs. postmenopausal controls (n=8)	88.8 89.9 vs. 144.9 106.6	Mann Whitney	Not sig.
Premenopausal prolapse (n=5) vs. postmenopausal prolapse (n=8)	112.4 95.7 vs. 89.9 90.1	Mann Whitney	Not sig.
Controls (n=13) vs. prolapse (n=13)	124.9 96.0 vs. 96.1 95.8	Wilcoxon	Not sig.
Premenopausal controls (n=5) vs. premenopausal prolapse (n=5)	88.8 89.9 vs. 112.9 96.0	Wilcoxon	Not sig.
Postmenopausal controls (n=8) vs. postmenopausal prolapse (n=8)	144.9 106.6 vs. 89.9 90.1	Wilcoxon	Not sig.

Summary of section 5.2:

Firstly, when comparing expression of compounds between premenopausal and postmenopausal populations, there were no significant differences in the expression of pro-MMP-2, pro-MMP-9, TIMP-2 and hydroxyproline content in vaginal skin samples from premenopausal and postmenopausal women in each group. However, active MMP-2 expression was significantly elevated in postmenopausal women with prolapse compared with premenopausal with prolapse were compared **($p<0.05$, Mann Whitney test).**

A significant increase in Pro-MMP-2 expression was found in samples of vaginal tissue from women with prolapse compared with controls **($p<0.01$, Wilcoxon matched paired, sign ranked test, $n=26$).**

There were small trends suggesting a rise in the expression of active MMP-2, pro-MMP-9 and TIMP-2 in vaginal skin from women with prolapse compared with controls. However, this was not statistically significant. Similarly there was no difference in the hydroxyproline content of vaginal skin from women with prolapse compared with controls, but a trend was seen suggesting vaginal tissue from premenopausal controls had more hydroxyproline than samples from premenopausal women with prolapse

5.3 Relationship between components in vaginal skin

The relationship between MMP-2 expression and TIMP-2 expression was evaluated. A positive relationship would suggest that as MMP-2 expression increases TIMP-2 expression also increases indicating remodeling of collagen rather than degradation. The results are outlined in tables 5.VII & 5.VIII, and significant results are in bold face and pictured in figures 5.9-5.12.

Table 5.VII ***Relationships between components from the premenopausal vaginal skin***

Relationship	**CONTROL (n=5)**			**PROLAPSE (n=5)**		
	Correlation Coefficient (r)	**Std deviation (Sy.x)**	**P value**	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**
Pro MMP-2 / TIMP-2	0.2557	6.122	0.678	-0.5598	2.277	0.326
Act MMP-2 / TIMP-2	0.7321	4.313	0.159	-0.7536	1.806	0.141
Hydoxyproline/ ProMMP-2	-0.2665	23.651	0.665	-0.319	45.478	0.599
Hydroxyproline/ Act MMP-2	-0.4905	21.383	0.4015	-0.113	47.693	0.856

Table 5.VIII ***Relationships between components from the postmenopausal vaginal skin***

Relationship	CONTROL (n=8)			PROLAPSE (n=8)		
	Correlation Coefficient (r)	Std deviation (Sy.x)	P value	Correlation Coefficient (r)	Std deviation (Sy.x)	P value
Pro MMP-2 / TIMP-2	0.5794	9.814	0.132	0.8533	6.416	**0.0071**
Act MMP-2 / TIMP-2	0.4434	10.793	0.271	0.9031	5.283	**0.0021**
Hydoxyproline/ ProMMP-2	-0.4030	84.692	0.322	0.8799	15.846	**0.004**
Hydroxyproline/ Act MMP-2	-0.0491	92.427	0.908	0.7251	22.965	**0.042**

No statistical relationships were found in the premenopausal vaginal skin. Statistically significant results were found for postmenopausal tissue. A positive relationship was found between the expression of pro-MMP-2 and the expression of TIMP-2 in samples of vaginal skin from postmenopausal women with prolapse (see figure 5.6). There is also a positive relationship between pro-MMP-2 expression and hydroxyproline content (see figure 5.7). A similar relationship was seen between active MMP-2 expression with TIMP-2 expression and hydroxyproline content (see figures 5.8 & 5.9).

Figure 5.6 ***Relationship between pro-MMP-2 and TIMP-2 expression in prolapse vaginal skin of postmenopausal women,*** **p<0.01, r=0.853**

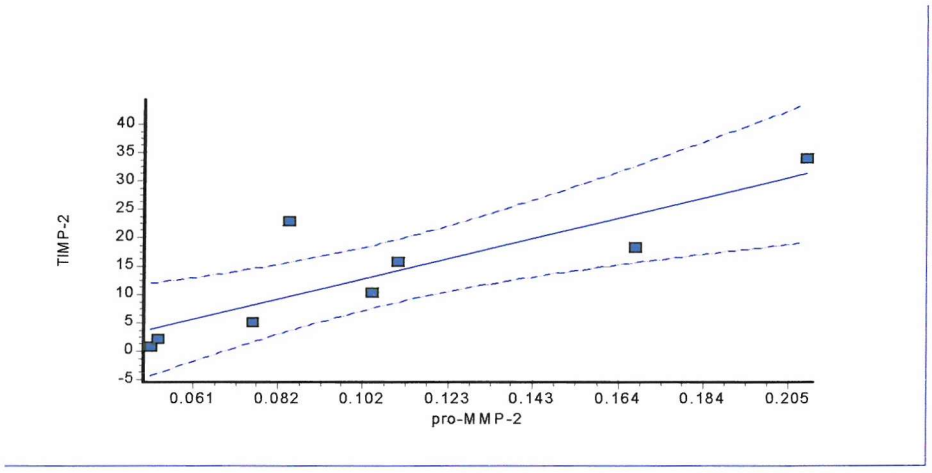


Figure 5,7 ***Relationship between pro-MMP-2 and hydroxyproline in prolapse vaginal skin of postmenopausal women,*** **p=<0.01, r= 0.879**

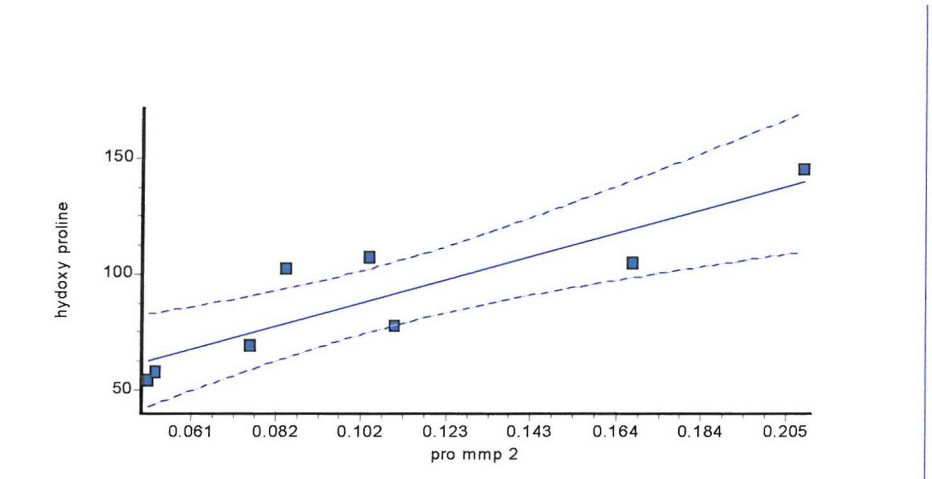


Figure 5.8 ***Relationship between active MMP-2 and TIMP-2 in prolapse vaginal Skin of postmenopausal women,*** **p<001, r=0.903**

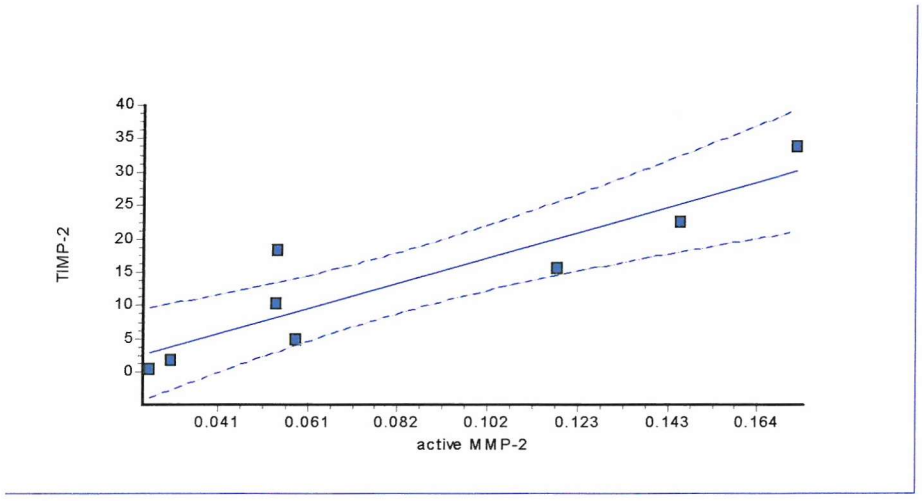
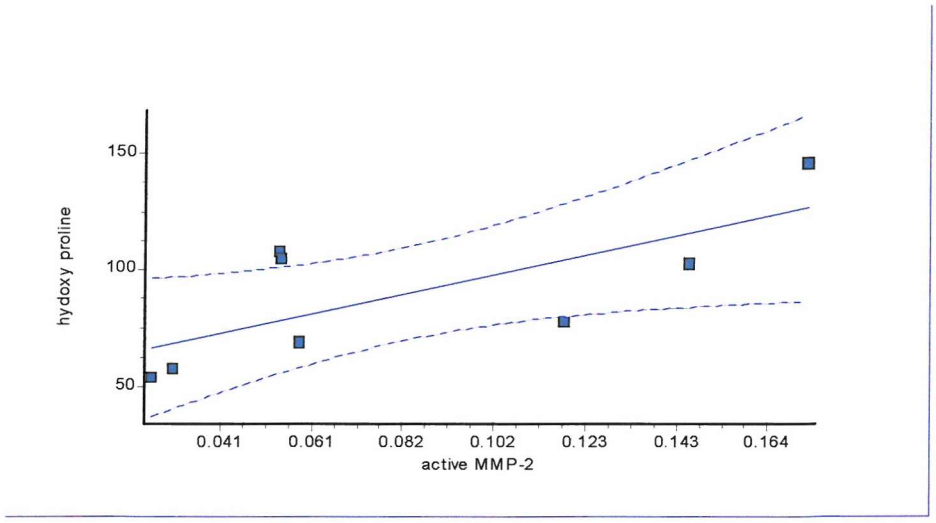


Figure 5.9 ***Relationship between active MMP-2 and hydroxyproline in prolapse vaginal skin of postmenopausal women,*** **p<0.05, r=0.725**



Summary of section 5.3:

There is a positive correlation between both pro and active MMP-2 expression with TIMP-2 expression in postmenopausal women with prolapse and controls. This was statistically significant in the postmenopausal women with prolapse ($p<0.01$: proMMP-2, $p<0.01$ active MMP-2). Similarly, in postmenopausal women with prolapse there was significant positive correlation between both pro and active MMP-2 expression and hydroxyproline content ($p<0.01$: proMMP-2, $p<0.05$: active MMP-2). Therefore, as MMP expression increases TIMP expression increases and the hydroxyproline content also rises in the tissue. This may suggest that tissue remodeling is taking place.

However, for women with prolapse in the premenopausal age group, there was a negative relationship between MMP expression and TIMP-2 activity although this was not statistically significant. This may suggest a different mechanism for the development of prolapse in this age group compared with postmenopausal women, such as tissue degradation rather than remodeling. There was no relationship between MMP expression and TIMP expression or hydroxyproline content in premenopausal controls or premenopausal women with prolapse.

PART 2: Collagen content and turnover in uterosacral ligaments

5.4 Differences in: pro-MMP-2, active MMP-2. pro-MMP-9, TIMP-2 and hydroxyproline expression in uterosacral ligament.

This is the first study to examine assays related to collagen metabolism in the uterosacral ligaments of women with prolapse compared with age / parity matched controls. Moreover, it is the first study to assess both MMP and TIMP expression in these tissues.

The two study groups have again been separated into premenopausal and postmenopausal populations and finally both populations together. The following tables show the mean and median values for pro-MMP-2, active MMP-2, pro-MMP-9, TIMP-2 and hydroxyproline found in samples taken from the uterosacral ligaments of women with prolapse and controls without prolapse. Results were initially analysed using the paired t-test as the data showed a normal distribution. However, results were also analysed using the Wilcoxon matched pair signed rank test, as the populations were small.

Table 5.IX ***Expression of pro-MMP-2, active MMP-2, pro-MMP-9, TIMP-2 and hydroxyproline in the uterosacral ligaments of premenopausal women with prolapse compared with controls without prolapse***

Assay	CONTROL (n=5)			VS. PROLAPSE (n=5)			P value for Wilcoxon test
	Mean	Median	Std Devn	Mean	Median	Std Devn	
Pro-MMP-2	0.0484	0.0483	0.0114	0.554	0.0561	0.0126	Not sig.
Active MMP-2	0.0348	0.0322	0.0253	0.0414	0.0378	0.0378	Not sig.
Pro-MMP-9	0.0276	0.0262	0.0216	0.0289	0.0281	0.0176	Not sig.
TIMP-2	5.80	6.72	2.81	5.71	5.37	4.52	Not sig.
Hydroxyproline	87.24	91.87	18.49	84.78	75.29	37.62	Not sig.

Table 5.IX demonstrates that in premenopausal women with prolapse there is a small increase in pro-MMP-2 and active MMP-2 expression within the uterosacral ligaments, compared with controls. However, this increase is only small and not statistically significant. There did not appear to be any significant difference in pro-MMP-9 expression between the two groups. There appears to be no difference in TIMP-2 expression in the uterosacral ligaments of the two groups, and hydroxproline concentration appears similar in the uterosacral ligaments of premenopausal women with prolapse compared with controls.

Table 5.X ***Expression of pro-MMP-2, active MMP-2, pro-MMP-9, TIMP-2 and hydroxyproline in the uterosacral ligaments of postmenopausal women with prolapse compared with controls without prolapse***

Assay	CONTROL (n=8)		VS.	PROLAPSE (n=8)			P value for Wilcoxn test
	Mean	Median	Std Devn	Mean	Median	Std Devn	
Pro-MMP-2	0.05655	0.0490	0.0243	0.0934	0.0604	0.0686	Not sig.
Active MMP-2	0.0245	0.0544	0.0086	0.0364	0.0642	0.0129	Not sig.
Pro-MMP-9	0.0221	0.0200	0.0118	0.0264	0.0264	0.0186	Not sig.
TIMP-2	3.305	3.140	2.275	10.046	8.315	8.440	**0.019** **0.004**
Hydroxyproline	83.16	80.80	25.37	74.10	70.00	19.11	Not sig.

Table 5.X shows that pro-MMP-2 and active MMP-2 levels are increased in the uterosacral ligaments of postmenopausal women with prolapse compared with controls without prolapse. However, this increase in expression was not statistically significant using the Paired t-test or the Wilcoxon MPSR test. There was no apparent difference in MMP-9 expression between the two groups. In coincidence with the small rise in MMP-2 expression, TIMP-2 activity was also found to be increased in the uterosacral ligaments of postmenopausal women with prolapse compared with controls. This was shown to be

statistically significant using the Wilcoxon MPSR test **($p<0.01$)**. These results would suggest that as in vaginal tissue of postmenopausal women with prolapse, there appears to be a rise in TIMP-2 expression which is coincident with MMP-2 expression. This may reflect tissue remodeling and new collagen formation rather than degradation. The results are less significant than seen in the vaginal skin of the same patients.

Summary of section 5.4:

In this section assays were performed on fascia taken from the uterosacral ligaments, rather than vaginal skin of women with prolapse and controls without prolapse.

Both pro-MMP-2 and active MMP-2 expression were increased in samples of uterosacral ligament from the women with prolapse. However, in contrast to samples of vaginal skin taken from the same women, the rise in pro MMP-2 and active MMP-2 were not statistically significant. There did not appear to be any difference in MMP-9 expression between the two groups.

There was a significant increase in TIMP-2 activity in the ligaments of postmenopausal women with prolapse compared with controls **($p<0.01$ by Wilcoxon test),** and a reduction in TIMP-2 activity in premenopausal women with prolapse compared with controls but was not statistically significant. There appeared to be no difference in hydroxyproline content between the two groups in either the premenopausl or postmenopausal populations. Again the relationship between pro-MMP-2 and TIMP-2 / hydroxyproline, active MMP-2 and TIMP-2 / hydroxyproline were investigated and outlined in the next section.

5.5 Relationship between components in the uterosacral ligaments

The relationship between MMP-2 expression and TIMP-2 expression was explored. If a positive relationship exists it would suggest that as MMP-2 expression increased TIMP-2 expression also increased indicating remodeling of collagen rather than degradation. The results are outlined in table 5.XI & 5.XII, and significant results are in bold face and pictured in figures 5.10 – 5.14.

Table 5.XI ***Relationships between tissue assays from the premenopausal uterosacral ligaments***

Relationship	**CONTROL (n=5)**			**PROLAPSE (n=5)**		
	Correlation Coefficient (r)	**Std deviation (Sy.x)**	**P value**	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**
Pro MMP-2 / TIMP-2	0.6020	2.590	0.283	-0.458	4.646	0.4378
Act MMP-2 / TIMP-2	0.3030	3.091	0.6202	-0.2967	4.992	0.6278
Hydoxyproline/ ProMMP-2	0.8245	12.084	0.0859	-0.4259	39.306	0.4746
Hydroxyproline/ Act MMP-2	0.7619	13.832	0.1344	-0.0050	43.442	0.9936

The previous table demonstrates there is a small negative relationship between pro-MMP-2 / active MMP-2 expression and TIMP-2 expression or hydroxyproline content. However, these results are not statistically significant.

Table 5.XII ***Relationships between tissue assays from the postmenopausal uterosacral ligaments***

Relationship	**CONTROL (n=8)**			**PROLAPSE (n=8)**		
	Correlation Coefficient (r)	**Std deviation (Sy.x)**	**P value**	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**
Pro MMP-2 / TIMP-2	-0.0629	2.452	0.882	0.8455	4.862	**0.0081**
Act MMP-2 / TIMP-2	-0.0075	2.457	0.9858	0.8438	4.893	**0.0085**
Hydoxyproline/ ProMMP-2	-0.7325	18.657	**0.0388**	0.7944	12.541	**0.0185**
Hydroxyproline/ Act MMP-2	-0.1497	27.095	0.7236	0.6963	14.818	0.055

This table outlines the results from the postmenopausal population. As can be seen in table 5.XII, there is a significant positive relationship between pro-MMP-2 expression and TIMP-2 expression in the uterosacral ligaments of postmenopausal women with prolapse **(n=8, r=0.8455, p<0.01)**, (see figure 5.10). This is associated with a positive relationship between pro-MMP-2 expression and hydroxyproline content **(n=8, r=0.7944, p<0.05)**,

(see figure 5.11). Similarly there was a significant relationship between active MMP-2 expression and TIMP-2 expression in the uterosacral ligaments of postmenopausal women with prolapse **(n=8, r=0.8438, p<0.01),** (see figure 5.12), along with a positive relationship between active MMP-2 expression and hydroxyproline content which did not quite reach statistical significance **(n=8, r=0.6963, p=0.055)**, (see figure 5.13). These data suggest that as MMP-2 expression increases in the uterosacral ligaments of postmenopausal women with prolapse, a coincident rise in TIMP-2 expression also occurs, leading to tissue remodeling and new collagen formation, which is seen by the associated rise in hydroxyproline content.

In postmenopausal controls without prolapse, there does not appear to be a positive correlation but negative correlation between MMP-2 expression and TIMP-2 expression / hydroxyproline content. These negative correlations were not statistically significant except for the relationship between active MMP-2 and TIMP-2 **(p=0.0388),** (see figure 5.14).

Figure 5.10 ***Relationship between pro-MMP-2 and TIMP-2 in uterosacral ligaments of postmenopausal women with prolapse, n = 8,r = 0.8459,*** **p <0.01**

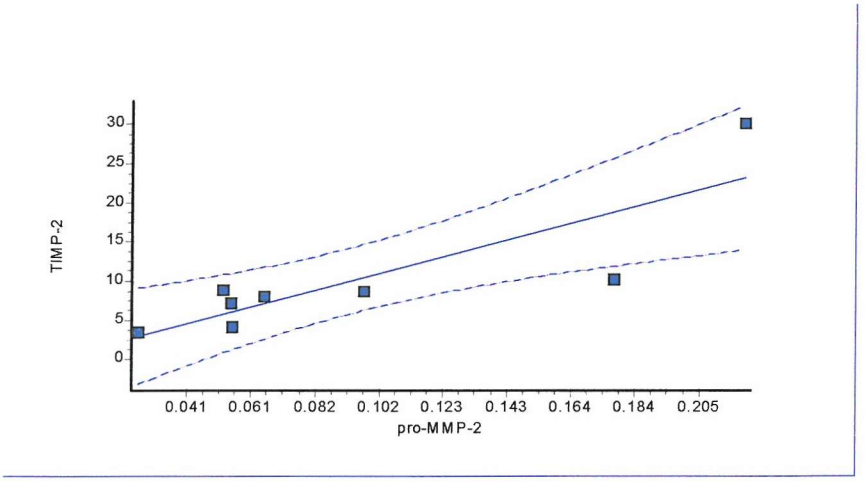


Figure 5.11 ***Relationship between pro-MMP-2 and hydroxyproline. in uterosacral ligaments of postmenopausal women with prolapse, n = 8,r = 0.7944,***

p <0.05

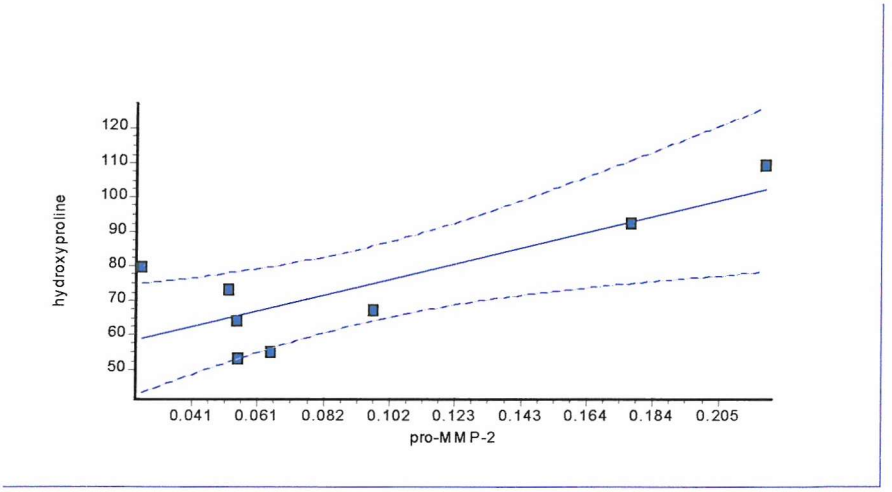


Figure 5.12 ***Relationship between active MMP-2 and TIMP-2 in uterosacral ligaments of postmenopausal women with prolapse, n = 8,r = 0.8438, p<0.01***

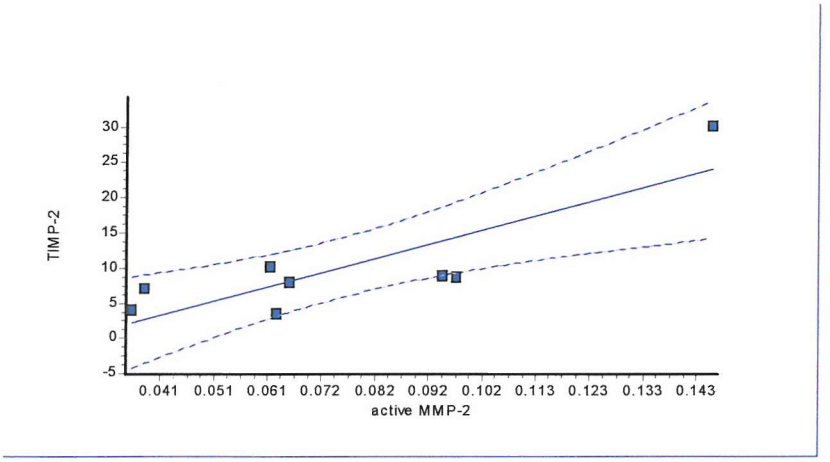


Figure 5.13 ***Relationship between active MMP-2 and hydroxyproline in uterosacral ligaments of postmenopausal women with prolapse, n = 8, r = 0.696, p = 0.0550 – not significant***

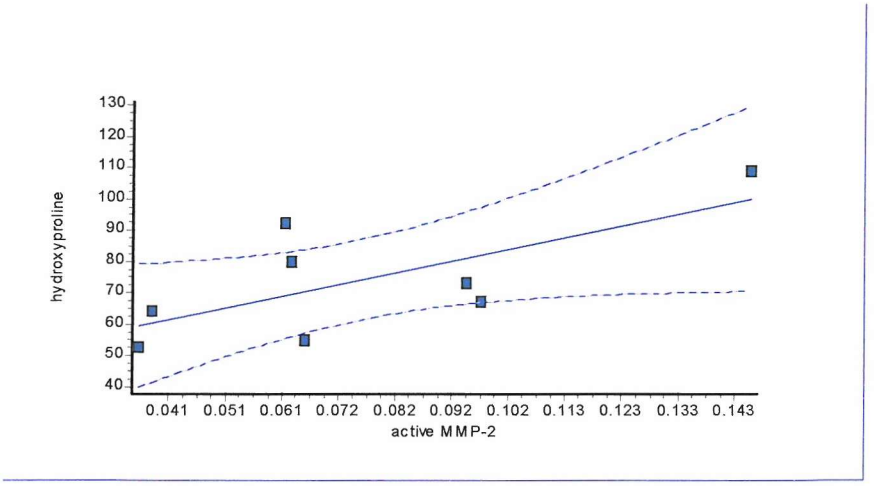
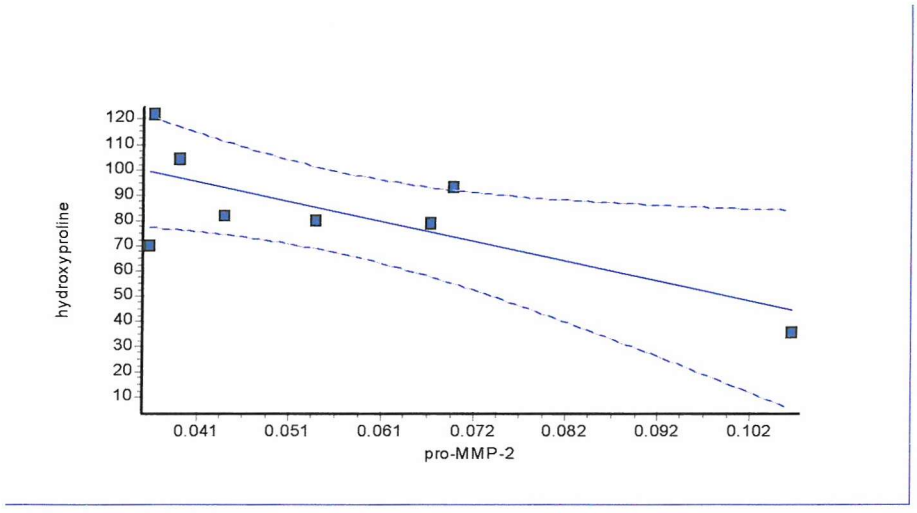


Figure 5.14 ***Relationship between pro-MMP-2 and hydroxyproline in uterosacral ligaments of control postmenopausal women, n = 8,r = -0.7325,*** **p <0.05**



Summary of section 5.5:

The relationship between MMP-2 expression and TIMP-2 or hydroxyproline content were determined in samples of uterosacral ligament taken from women with prolapse compared with similar samples of uterosacral ligament taken from age matched controls.

In control patients of both age groups, there was no relationship between pro and active MMP-2 expression with TIMP-2 expression. There was a negative relationship between MMP-2 expression and hydroxyproline content in ligaments of postmenopausal controls which was statistically significant for the pro-enzyme **($p<0.05$).** For premenopausal controls there did not appear to be a significant relationship between MMP-2 expression and TIMP-2 expression or hydroxyproline content.

However, in patients with prolapse a totally different relationship was noted. In postmenopausal women with prolapse there was a significant positive relationship between pro and active MMP-2 expression with TIMP-2 expression (**$p<0.01$**: pro MMP-2 and TIMP-2, **$p<0.01$**: active MMP-2 and TIMP-2). Moreover there was a positive relationship between pro and active MMP-2 expression with hydroxyproline content in postmenopausal women (**$p<0.01$**: pro MMP-2 and hydroxyproline, $p=0.055$: active MMP-2 and hydroxyproline). In premenopausal women with prolapse there appeared to be a negative relationship between MMP-2 expression and TIMP-2 expression /hydroxyproline content, however, these results did not reach statistical significance.

PART 3: Relationship between collagen metabolism in the vaginal skin and in the uterosacral ligaments

5.6 Relationship between components in vaginal skin and in uterosacral ligament.

The aim in this study was to determine if changes in collagen content and metabolism in the vaginal tissues reflect similar changes in the uterosacral ligaments. The relationship between the expression of each component in vaginal skin and the uterosacral ligament was examined. There was a significant positive correlation for the expression of pro-MMP-2, active MMP-2, Pro-MMP-9 and TIMP-2 **($p<0.0001$, $p<0.0001$, $p<0.01$ and $p<0.01$** respectively). See figures 5.15-5.18. There was no correlation for hydroxyproline content.

Figure 5.15 Correlation between pro-MMP-2 expression (AU/mg total protein) in the vaginal skin with uterosacral ligament ($n=26$, $r=0.83$, $p<0.0001$)

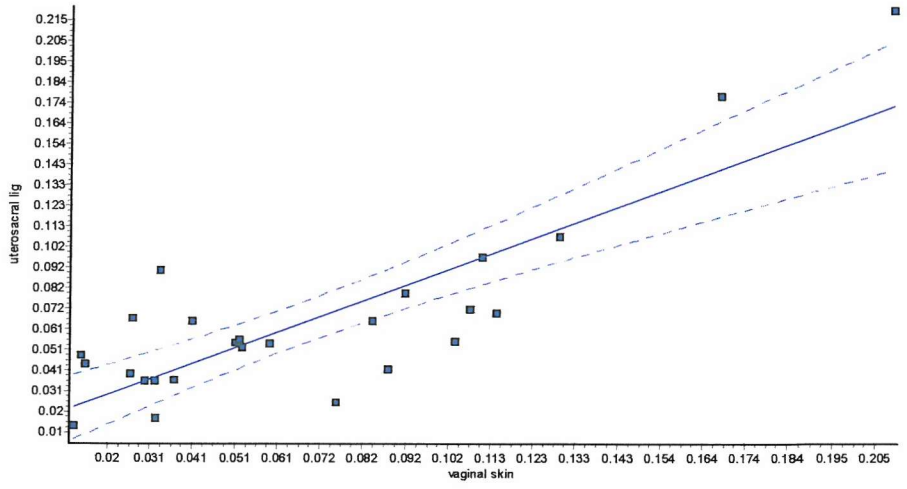


Figure 5.16 Correlation between active-MMP-2 expression (AU/mg total protein) in the vaginal skin with uterosacral ligament (n=26, r=0.69, p<0.0001)

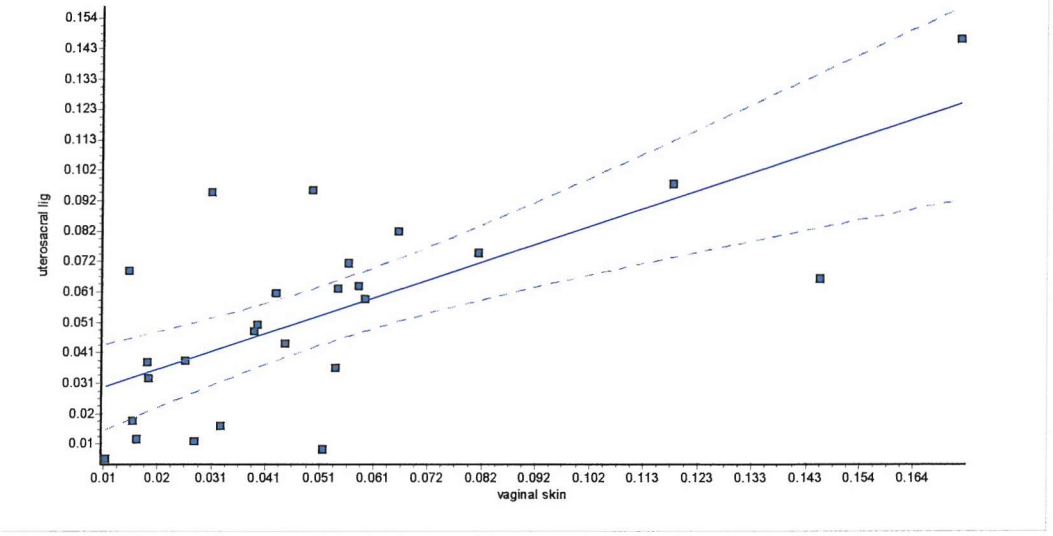


Figure 5.17 Correlation between pro-MMP-9 expression (AU/mg total protein) in the vaginal skin with uterosacral ligament (n=26, r=0.55, p<0.01)

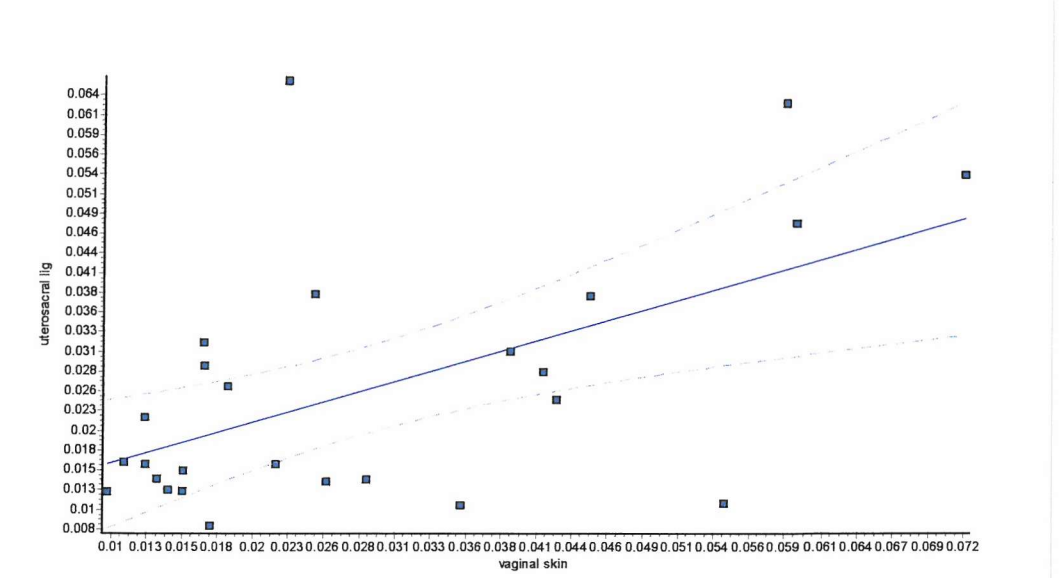
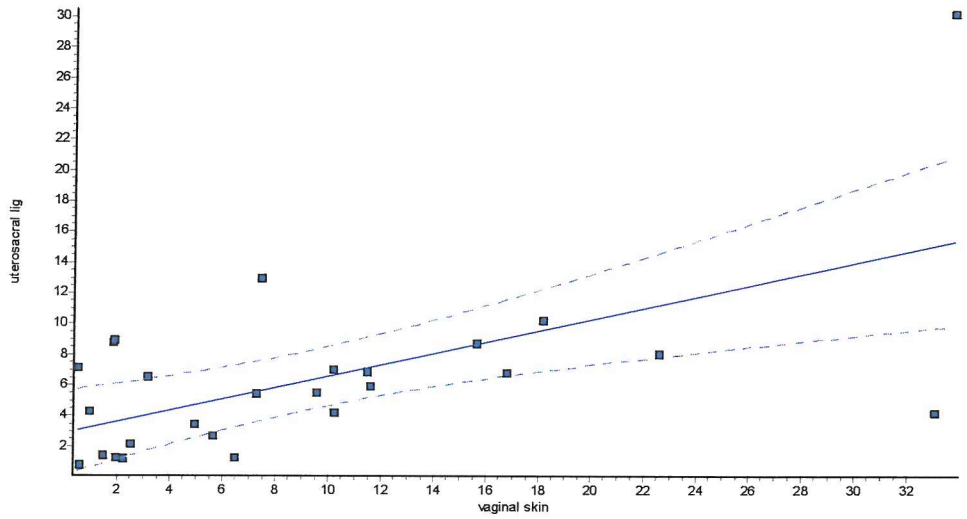


Figure 5.18 Correlation between TIMP-2 expression ng/mg protein) in the vaginal skin with uterosacral ligament (n=26, r=0.59, p<0.01)



As in the previous parts of chapter 5, the whole groups were further analysed after being divided into premenopausal and postmenopausal populations. The results are outlined in tables 5.XIII and 5.XIV (significant results are type-faced in bold).

Table 5.XIII Correlation between components in vaginal skin and the uterosacral ligament in premenopausal women

	CONTROL (n=5)			PROLAPSE (n=5)		
Relationship between vaginal skin and ligament	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**
Pro-MMP-2	0.8089	0.0174	0.0973	0.1261	0.0323	0.09
Active MMP-2	0.8818	0.0138	**0.0479**	0.4676	0.0385	0.4271
MMP-9	0.9303	0.0091	**0.0219**	0.6792	0.0149	0.207
TIMP-2	-0.01343	3.243	0.983	0.6.13	2.195	0.283
Hydroxyproline	0.1787	21.01	0.774	-0.2779	41.731	0.6508

This table shows that there is a positive correlation between the expression of pro-MMP-2 in the vaginal skin and uterosacral ligaments of premenopausal controls and premenopausal women with prolapse. This was almost reached statistical significance in the premenopausal women with prolapse ($p=0.09$). Again there was a positive relationship for the expression of active MMP-2 and pro-MMP-9 in premenopausal women with prolapse and controls. This reached statistical significance for the control group (**$p<0.05$**; active MMP-2, **$p<0.05$** pro-MMP-9), but not the women with prolapse. There were no significant correlations for TIMP-2 expression and hydroxyproline content.

Table 5.XIV Correlation between components in vaginal skin and the uterosacral ligament in postmenopausal women

	CONTROL (n=8)			PROLAPSE (n=8)		
Relationship between vaginal skin and ligament	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**	**Correlation Coefficient (r)**	**Std deviation (Sy.x)**	**P value**
Pro-MMP-2	0.8244	0.0149	**0.0118**	0.9439	0.024	**0.0004**
Active MMP-2	0.6131	0.0209	0.106	0.6689	0.02924	0.0697
MMP-9	0.944	0.0042	**0.0004**	-0.069	0.02006	0.8705
TIMP-2	0.4894	2.143	0.2183	0.7641	7.938	**0.0273**
Hydroxyproline	0.2771	23.3	0.1802	0.4739	18.181	0.2356

There was a significant positive correlation for pro-MMP-2 expression in the vaginal skin and in the uterosacral ligaments of postmenopausal controls and postmenopausal women with prolapse (**$p<0.05$ and $p<0.001$** respectively), and for proMMP-9 expression in postmenopausal controls **($p<0.001$).** There was also a positive correlation between active MMP-2 expression within the vaginal skin and within the uterosacral ligaments but these did not reach statistical significance. There was a significant positive correlation between TIMP-2 expression in the vaginal skin and in the uterosacral ligaments of postmenopausal women with prolapse (**$p<0.05$**).

Summary of section 5.6:

Although statistically significant differences in components were seen in vaginal tissue from women with prolapse compared with controls, no statistically significant results were seen between the two groups when analyzing component levels in the uterosacral ligaments. The question therefore arose whether the changes seen in the vaginal tissue were representative of changes in collagen metabolism in the uterosacral ligaments. The correlation between the expression of components in the vaginal skin with their expression in the uterosacral ligaments was studied.

There was a significant correlation between the expression of pro-MMP-2, active MMP-2, pro-MMP-9 and TIMP-2 within the two tissue types (**$p<0.0001$, $p<0.0001$, $p<0.01$, $p<0.01$ respectively**). A positive correlation in hydroxyproline content was seen but was not statistically significant. These results suggest that the vaginal tissue does reflect changes in MMP and TIMP activity in the uterosacral ligaments, but that these changes are more profound in the vaginal tissue.

Correlations became less statistically significant when numbers were reduced by dividing the whole population of women (n=26) into study groups and premenopausal and postmenopausal populations.

RESULTS

Chapter 6:

Histology of the uterosacral ligament

Contents to Chapter 6:

CHAPTER 6:

Histology of the uterosacral ligament

5μm thick sections of tissue taken from the uterosacral ligaments of women with prolapse and controls.were cut and used for immunostaining. Immunostaining was achieved using a three-layer technique. A typical section through the uterosacral ligament would have areas of connective tissue with various blood vessels and nerves cut in cross-section. Interspersed between the areas of connective tissue are segments of smooth muscle. This is in keeping with the histology noted by Campbell (1950). The smooth muscle fibres are arranged in bundles but there does not appear to be any specific orientation. Within the same section there are bundles of smooth muscle cut along their longitudinal axis as well as in cross section (see fig 6.1). The connective tissue element was very fibrous in nature and often would not adhere well to the APES coated slides.

6.1 Oestrogen receptors

Sections were stained with the monoclonal mouse anti-human oestrogen receptor antibody; clone ID5 (isotype IgG1, kappa) as per the technique used by Bernstein, (1997). Serial sections used for negative controls were stained with a non specific monoclonal mouse antibody of the same isotype (IgG1, kappa) diluted to the same concentration as the

ID5 antibody. Positive control slides were made from sections of breast carcinoma stained with ID5.

As demonstrated in figures 6.1 and 6.2 the ID5 monoclonoal antibody is very specific and only stains the nuclei of the smooth muscle cells of the uterosacral ligaments. No positive staining was found in the nuclei of smooth muscle cells in the blood vessels that pass through the uterosacral ligament. This shows the oestrogen receptor is specifically present in the smooth muscle bundles but not in all smooth muscle. This is similar to findings by Mokrzycki et al,(1997).

Oestrogen receptor expression was quantified by calculating the number of positively stained nuclei per 1000 smooth muscle cell nuclei. All nuclei counted had to have the specific morphology of a smooth muscle nucleus. Results are expressed as percentages of the total number (1000) of nuclei .

There was no significant difference in the proportion of positively stained nuclei in the uterosacral smooth muscle of women with prolapse compared with age-matched controls in the premenopausal or postmenopausal populations, or when pre and postmenopausal populations were combined (paired t test, Wilcoxon matched pairs signed rank test). There was no difference in oestrogen receptor expression before and after the menopause (Mann Whitney test) and no correlation between age of controls and oestrogen receptor expression.

Figure 6.1 Section through uterosacral ligament demonstrating blood vessels (BV), nerves (N), smooth muscle bundles (SM) and connective tissue (CT)

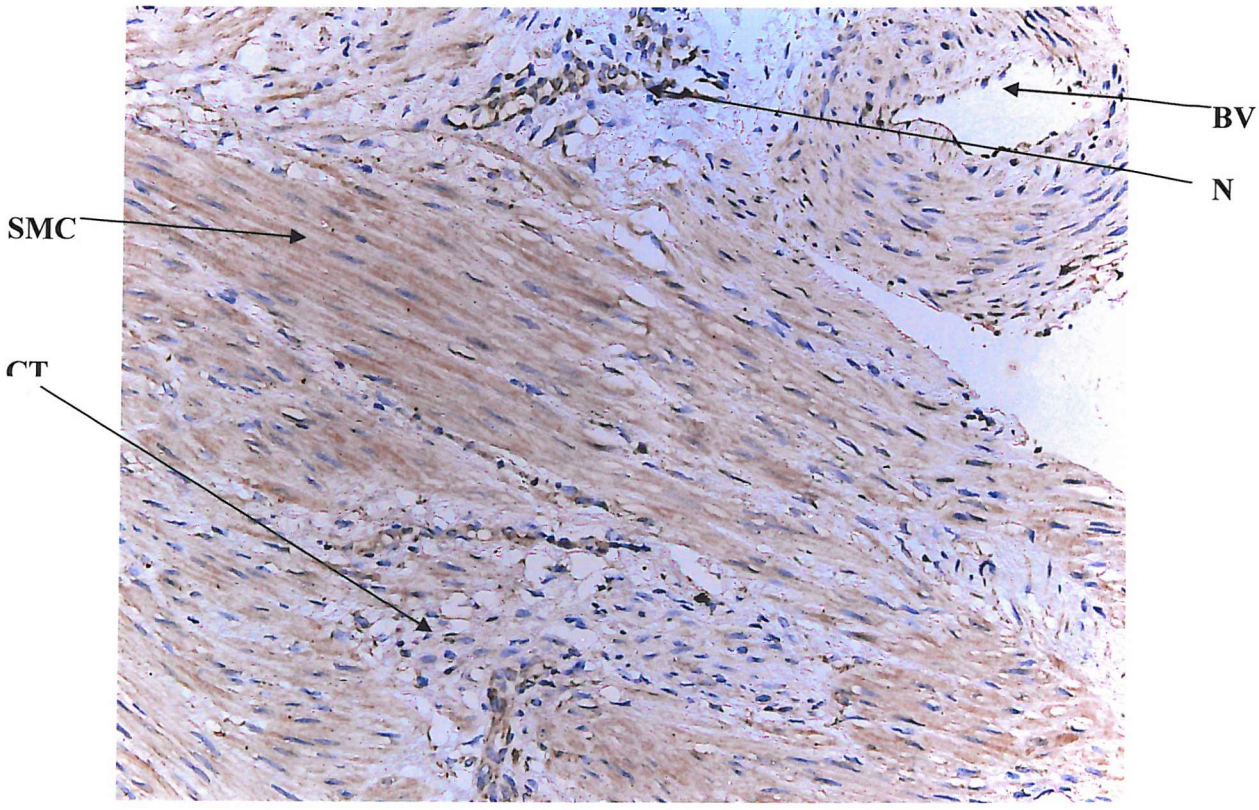
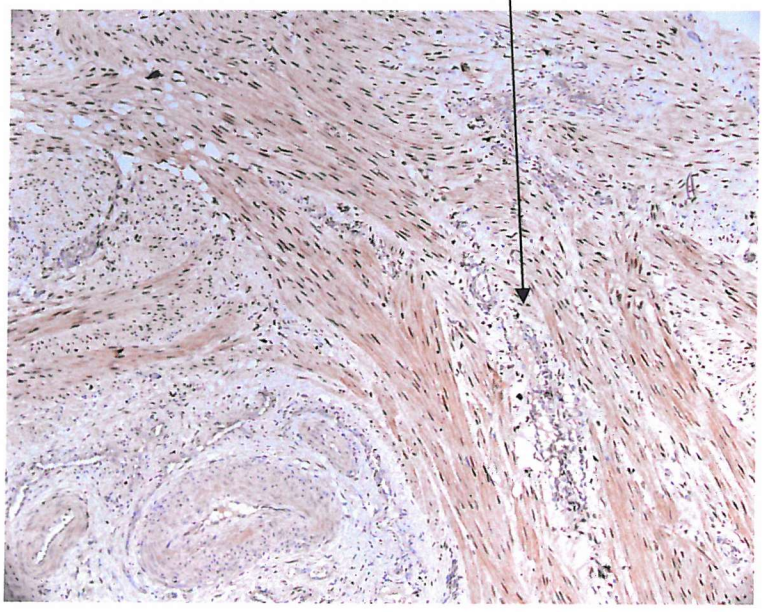
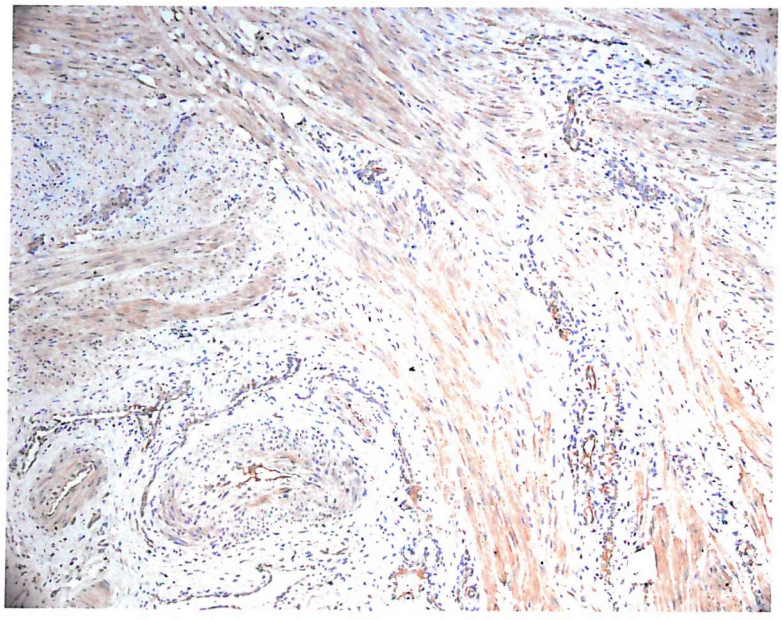


Figure 6.2: ***Section Uterosacral ligament stained with monoclonal antibody ID5 demonstrating the oestrogen receptor (OR) in the smooth muscle cell bundles but not in the smooth muscle of the blood vessels. Below a serial section negative control, stained with non-specific mouse IgG1 kappa***



Strong staining of nuclei, with minimal background staining of cytoplasm.

This demonstates oestrogen receptor

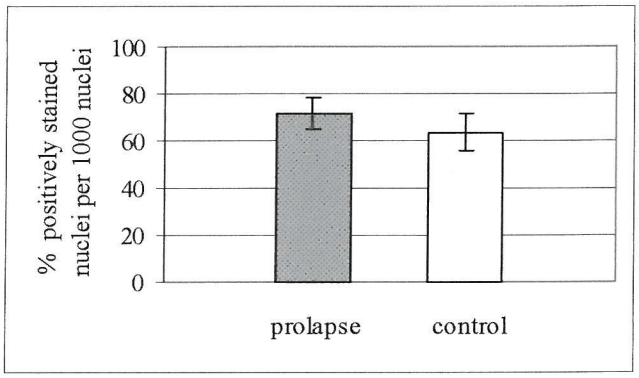


Negative control

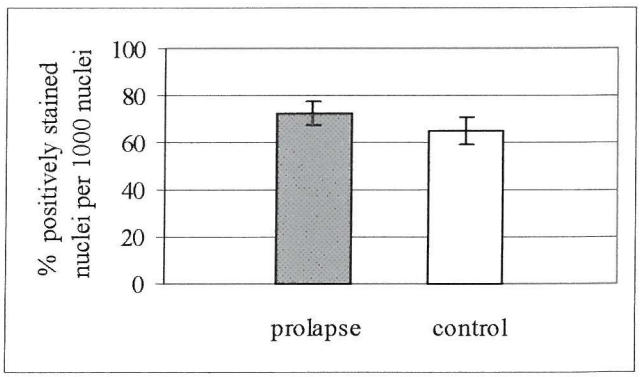
Serial section stained with non specific mouse IgG1 kappa (DAKO)

Figure 6.3 ***Percentage of positively stained smooth muscle nuclei in the uterosacral ligaments of women with prolapse compared with controls. (mean +/- SEM)***

Postmenopausal women **Not significant**

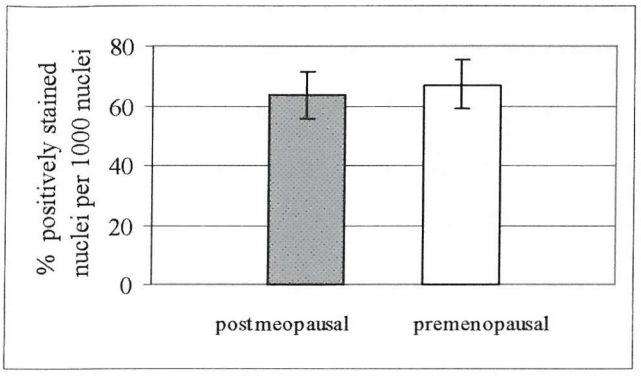


Premenopausal women **Not significant**



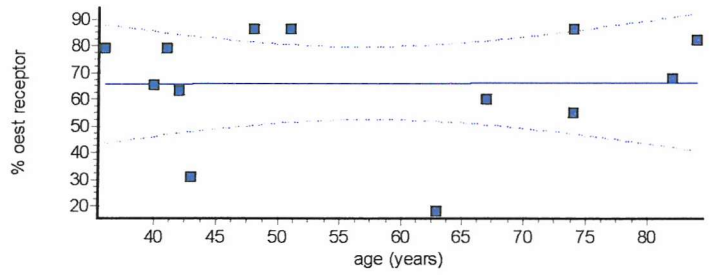
There are no significant differences between the number of positively stained nuclei in the prolapse and control groups for either the premenopausal or postmenopausal populations, as tested using the Wilcoxon matched paired, sign ranked test.

Figure 6.4 ***Comparison of oestrogen receptor expression in the uterosacral ligaments of pre and postmenopausal controls. (mean +/-SEM)***



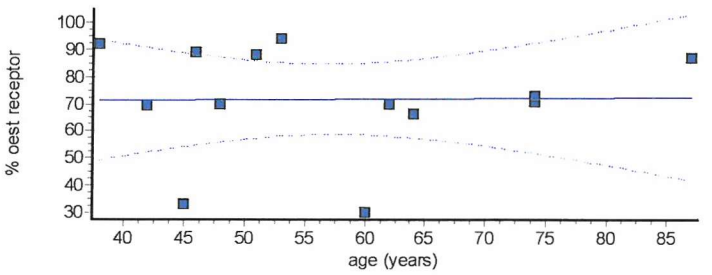
Not significant

Figure 6.5 ***Correlation between age (years) and oestrogen receptor expression (% positively stained nuclei) in patients with prolapse & controls (excluding patients on HRT)***



Controls

Not significant

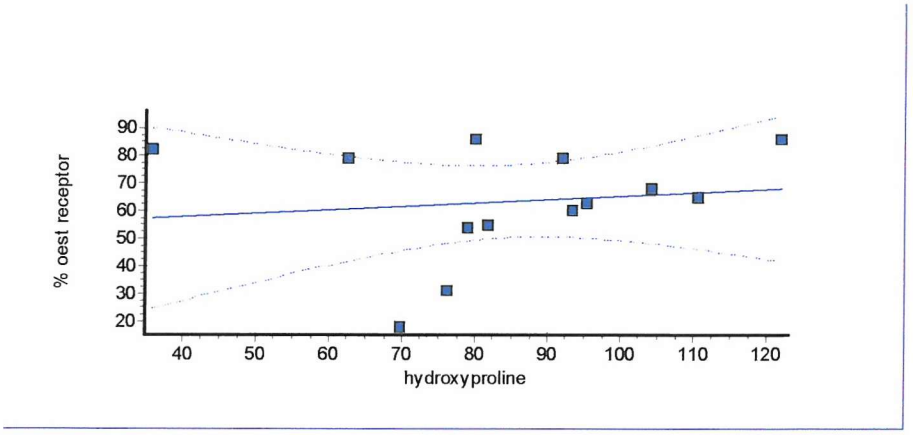


Prolapse

Not significant

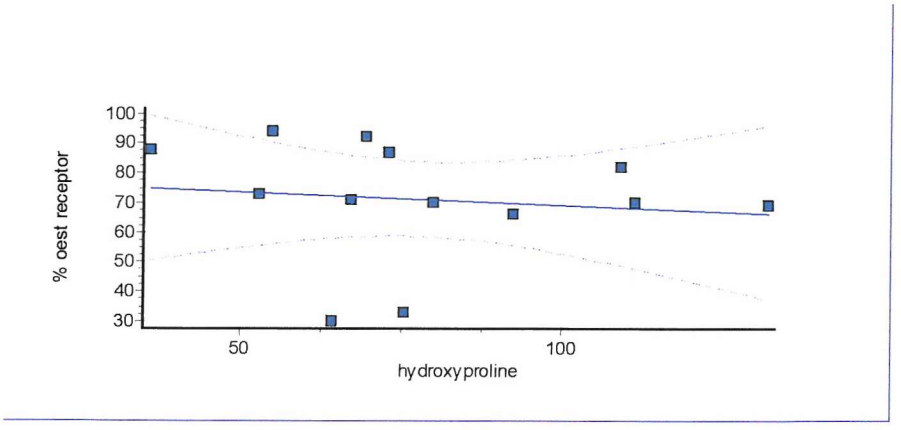
There was no relationship between oestrogen receptor expression in the smooth muscle bundles of the uterosacral ligaments and the hydroxyproline content of the uterosacral ligaments in controls or women with prolapse as demonstrated in figure 6.6.

Figure 6.6 Relationship between oestrogen receptor expression (% positively stained nuclei) and hydroxyproline concentration in the uterosacral ligaments of women with prolapse compared with controls



Controls

Not significant



Prolapse

Not significant

Summary to section 6.1:

Oestrogen receptors are present within the nuclei of smooth muscle bundles in the uterosacral ligaments, but not in smooth muscle of blood vessels. This was similar to findings in the study by Mokrzycki et al (1997). No studies have tried to quantify the oestrogen receptor expression within the uterosacral ligaments. No correlation was found between the age of women and the expression of oestrogen receptors within the smooth muscle nuclei of their uterosacral ligaments for the control group. In addition there was no correlation in women with prolapse. There was no significant difference in the expression of smooth muscle oestrogen receptors in premenopausal controls compared with postmenopausal controls. There was no difference in oestrogen receptor expression in women with prolapse compared with age matched controls. Finally, there was no relationship between smooth muscle oestrogen receptor expression and the hydroxyproline content in the uterosacral ligaments of women with prolapse or controls.

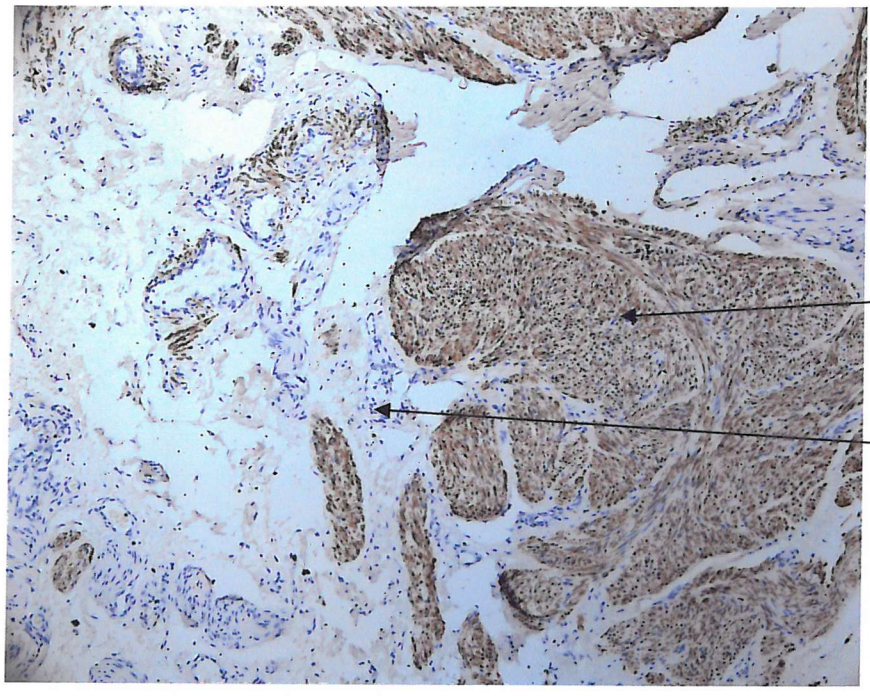
The presence of the oestrogen receptor within the smooth muscle of the uterosacral ligament suggests that it may be a target for treatment of prolapse. However, there did not appear to be a demonstrable difference in the quantity of oestrogen receptor expression in women with prolapse compared with controls, and therefore differences may exist either upstream or downstream to the receptor.

6.2 Smooth muscle cell phenotype

Sections of uterosacral ligament were stained with mouse monoclonal antibodies specific to the smooth muscle / myofibroblast antigens *vimentin, desmin* and *α-smooth muscle actin*. Negative control slides were stained with non specific mouse monoclonal antibodies *IgG1,IgG1* and *IgG2α* respectively. Positive control slides of human colon were used to demonstrate positive staining.

There was no background staining with any of the non-specific antibodies. Antibodies to vimentin, desmin and *α-smooth muscle actin* all positively stained for all smooth muscle cells: both in the uterosacral ligament bundles as well as in the walls of blood vessels. There was no staining of any other structures other than the smooth muscle cell fibres. Areas of smooth muscle were chosen where the fibres were orientated longitudinally. In these areas the density of staining for each antigen was assessed using a computerised image analysis package. A calibration density slice was performed on a control slide of colon prior to each analysis to ensure reproducibility of results (coefficient of variation 16%). Results are expressed as the mean of five separate areas of smooth muscle analysed for each patient.

Figure 6.7 Section of smooth muscle stained for desmin and a serial section stained with the negative control (non-specific mouse IgG1 kappa)

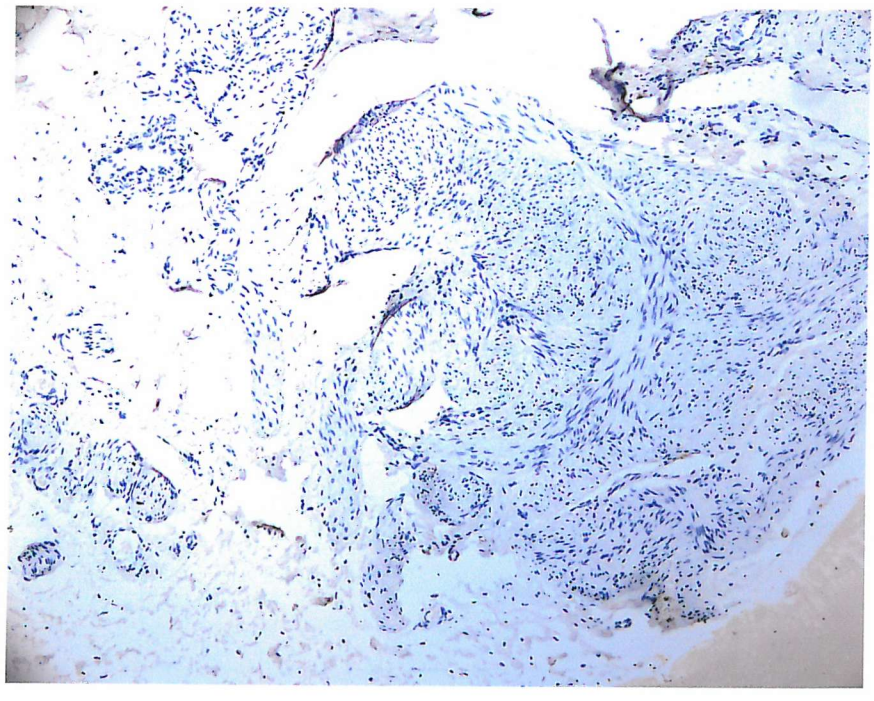


Section stained with mouse monoclonal anti-human desmin antibody.

X10 magnification

Positive areas of smooth muscle cytoplasm staining brown.

Connective tissue which doesn't stain.



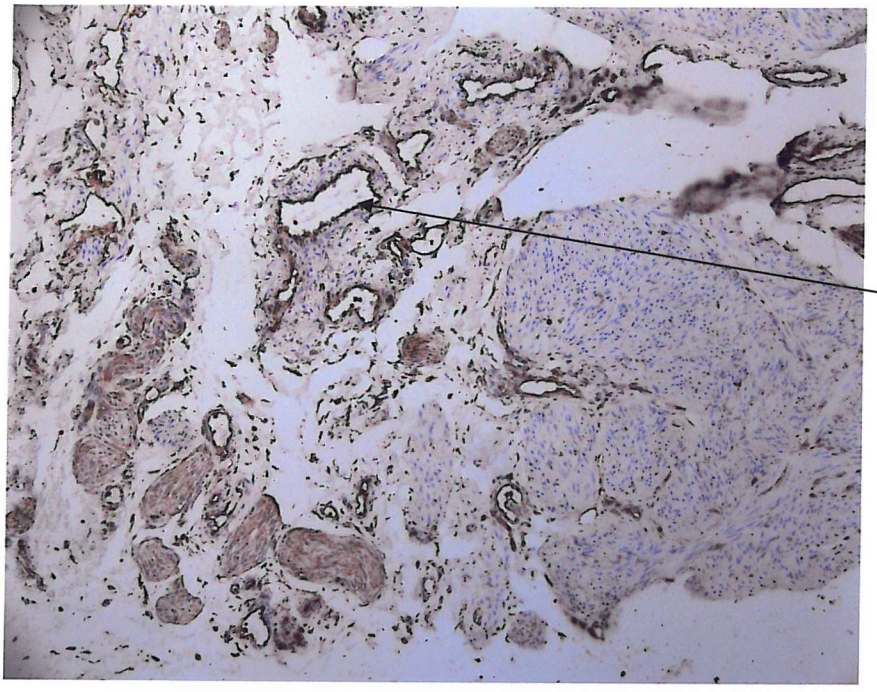
NEGATIVE CONTROL

X10 magnification

Section stained with non specific mouse IgG1, kappa.

Notice no positive staining of the smooth muscle.

Figure 6.8 *Section of smooth muscle stained for vimentin and a serial section stained with the negative control (non-specific mouse IgG2α, kappa)*

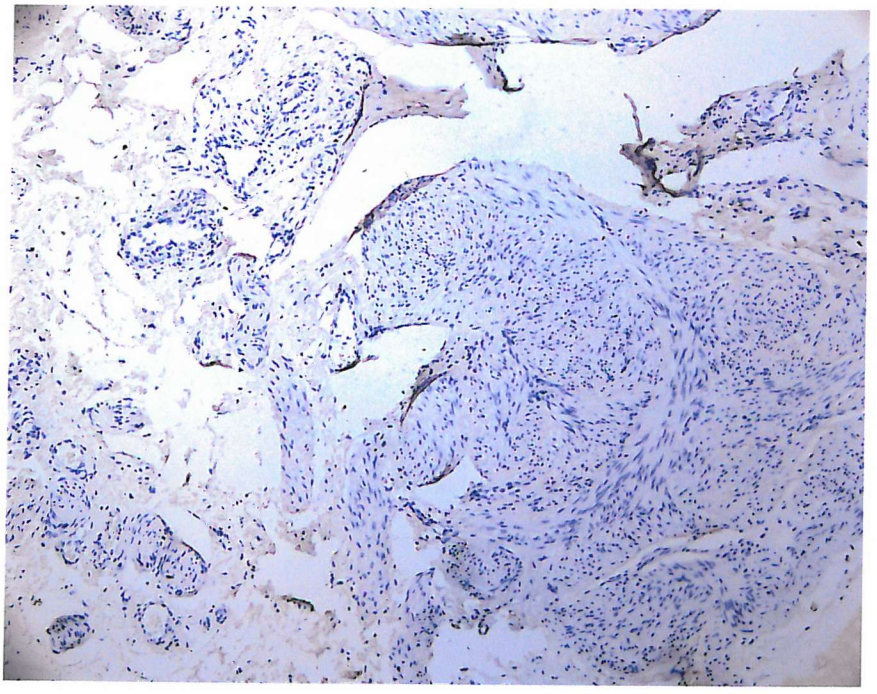


Section stained with mouse monoclonal anti-human vimentin antibody.

X10 magnification

Positive areas of vascular smooth muscle, with minimal uterosacral muscle staining.

Connective tissue which doesn't stain.



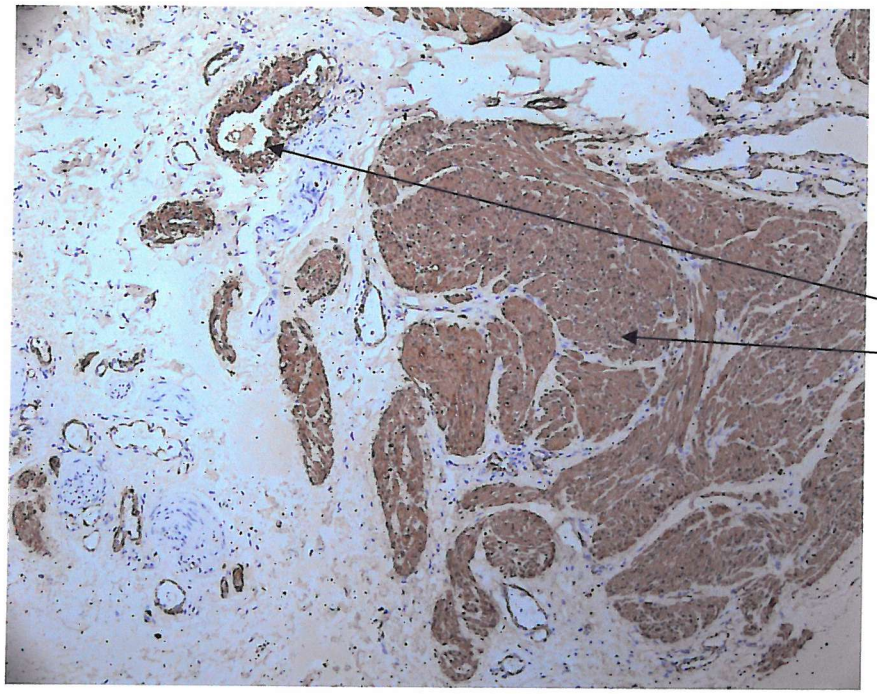
NEGATIVE CONTROL

X10 magnification

Section stained with non specific mouse IgG2α, kappa.

Notice no positive staining of the smooth muscle.

Figure 6.9 ***Section of smooth muscle stained for α-smooth muscle actin and a serial section stained with the negative control (non-specific mouse IgG2α, kappa)***

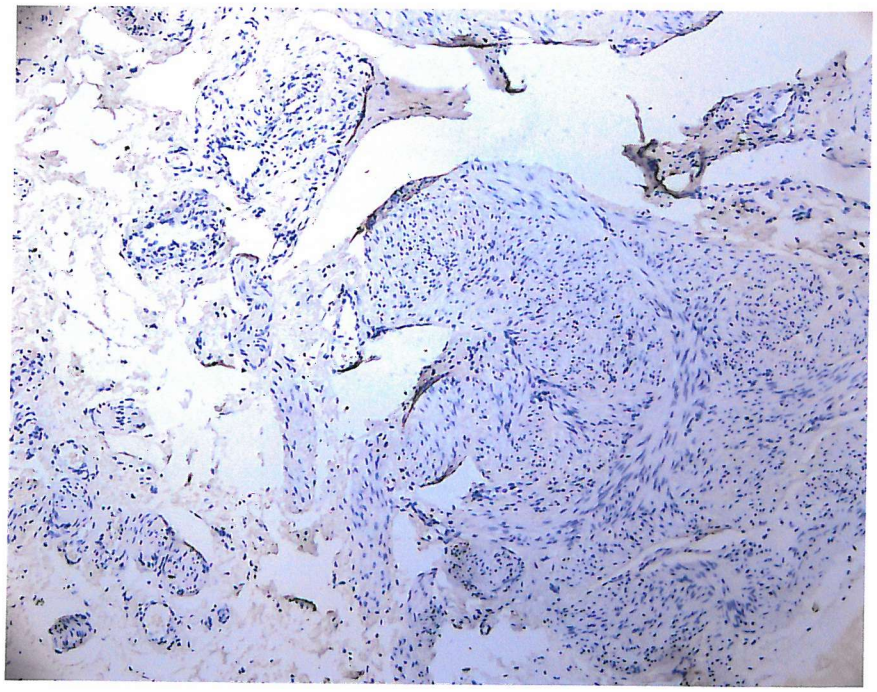


Section stained with mouse monoclonal anti-human α-smooth muscle actin antibody.

X10 magnification

Positive areas of vascular smooth muscle,and uterosacral muscle staining significantly.

Connective tissue which doesn't stain.



NEGATIVE CONTROL

X10 magnification

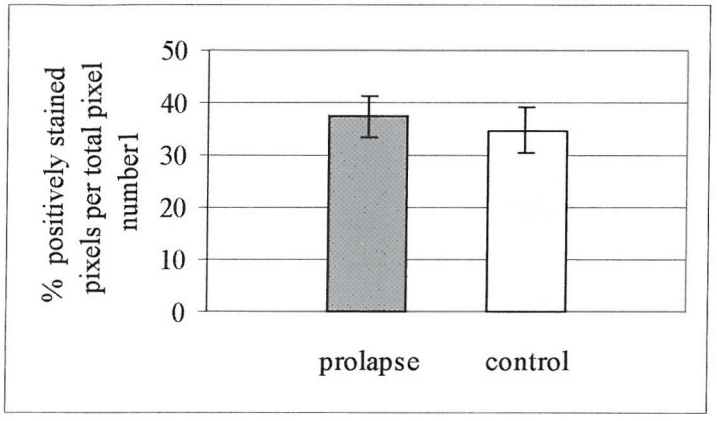
Section stained with non specific mouse IgG2α, kappa.

Notice no positive staining of the smooth muscle.

By densitometry analysis, there was no significant difference in the density of staining for desmin, vimentin or α-smooth muscle actin in the fibres of uterosacral ligament smooth muscle bundles of women with prolapse compared with controls using the Wicoxon matched paired, sign ranked test (see figures 6.10-6.12).

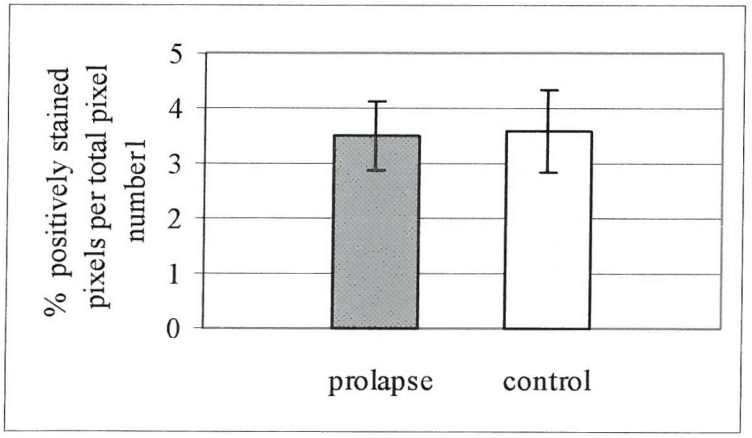
There was evidence of staining for all three phenotypes within the tissue sections. There was a high density of staining of α-smooth muscle actin in both the uterosacral ligament smooth muscle (60%) but also in high quantities in the smooth muscle around blood vessels. Staining was predominantly confined to the cytoplasmic elements with nuclear sparing. Desmin was predominantly found in the cytoplasm of the uterosacral ligament smooth muscle rather than vascular smooth muscle, but less pronounced than α-smooth muscle actin (35% vs 60%). In contrast to the dense positive staining for desmin and α-smooth muscle actin, vimentin did stain most samples but the density of expression was much less in the uterosacral ligament smooth muscle compared with the other markers. (3%), but was more pronounced in the vascular smooth muscle compared with the uterosacral ligament smooth muscle.

Figure 6.10 Density of staining for desmin (percentage of positively stained pixels per total pixel number) in uterosacral smooth muscle bundles of women with prolapse compared with control (mean +/-SEM)



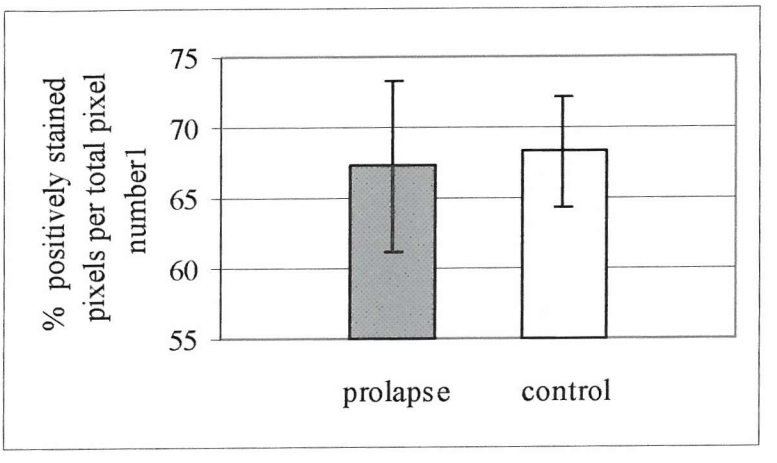
Not significant

Figure 6.11 Density of staining for vimentin, (percentage of positively stained pixels per total pixel number) in uterosacral smooth muscle bundles of women with prolapse compared with controls, (mean +/-SEM)



Not significant

Figure 6.12 ***Density of staining for α-smooth muscle actin, (percentage of positively stained pixels per total pixel number) in uterosacral smooth muscle bundles of women with prolapse compared with controls.(mean +/-SEM)***



Not significant

Summary to section 6.2:

The smooth muscle / myofibroblast antigens vimentin, desmin and α-smooth muscle actin were all present in the smooth muscle bundles of the uterosacral ligaments. α-smooth muscle actin was found in the smooth muscle of the ligaments and blood vessels, whereas desmin was predominantly in the smooth muscle bundles and vimentin in the vascular smooth muscle. There was no significant difference in the expression of each antigen in women with prolapse compared with controls.

These results suggest that the smooth muscle cells of the uterosacral ligament adopt a smooth muscle phenotype rather than myofibroblast. The relative proportions of phenotypes do not appear to change in women with prolapse compared with controls, but may be still be implicated in the production of metalloproteinases.

DISCUSSION

Chapter 7:

Discussion

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7.1 Collagen metabolism in the vaginal skin

Many theories have been proposed for the aetiology of prolapse and genuine stress incontinence. These include muscular injury that occurs during parturition (Debus-Tiede, 1993) and denervation to the muscles of the pelvic floor (Snooks et al, 1984, 1986, 1990). However, this doesn't explain why prolapse effects 2% of nulliparous women, as well as women who have been through childbirth but have retained normal neuromuscular function. This led to the study of the endopelvic fascia and in particular the strongest constituent of fascia: collagen.

It has been suggested that abnormalities in the metabolism of collagen found within vaginal fascia are due to defects in collagen metabolism that is inherent throughout the whole body (Norton et al, 1990). Numerous workers have focused on the properties and metabolism of collagen in the vaginal epithelium and in particular the fascia immediately deep to the vaginal epithelium. This is probably due to the relative ease with which these biopsies can be harvested. In doing so, workers have extrapolated that vaginal biopsies reflect tissue within the endopelvic fascia. One small pilot study seemed to suggest this (Keane 1993). However, the histology of the supportive structures of the uterus: the paracolpium, uterosacral and cardinal ligaments, demonstrates these structures have totally different composition compared with the vaginal epithelium. The aim of this study was to investigate the changes in hydroxyproline content, metalloproteinase 2 and 9 expression as

well as TIMP-2 expression within the vaginal skin in relation to the uterosacral ligaments, in women with prolapse and a control group of women.

A significant increase in the expression of pro-MMP-2 was found in the vaginal tissue of women with prolapse ($p<0.01$, $n=26$). This was similar to the findings of Jackson et al, (1996). A small increase in active MMP-2 was seen in vaginal tissue from women with prolapse but this was not statistically significant. Only a single band was found in the region that corresponded to the molecular weight of pro-MMP-9. A separate band in the molecular weight region of the active form of the enzyme was not seen. Semi-quantification of this band showed a small but not significant increase in the concentration of pro-MMP-9 within biopsies of vaginal epithelium from patients with prolapse. The results for active MMP-2 and pro-MMP-9 differed slightly from the findings of Jackson et al. They too could not demonstrate a separate band for active MMP-9 but did show an increase in the pro-enzyme which just reached significance ($p=0.048$) and a significant rise in active MMP-2 ($p=0.048$). There may be two reasons to account for the discrepancy. Firstly, the patients in the present study were of both pre and postmenopausal ages. The women studied by Jackson et al, were only premenopausal and were matched for their age and parity, and not matched for the phase in their menstrual cycle. Although MMP activity alters within the endometrium during the menstrual cycle to play an important role in menstruation (Singer et al, 1997; Courtoy et al, 1998), workers have shown that oestradiol can stimulate the release of MMP-2 from cells distant from the endometrium (Wingrove et al, 1998). As circulating oestradiol may affect release of metalloproteinases in extrauterine sites such as the vaginal skin and endopelvic fascia, all

patients in this study were matched for hormonal status, duration of hormone replacement therapy and phase of menstrual cycle. Secondly Jackson et al, extracted tissue using a tissue dismembranator and added a variable volume of buffer to give a constant concentration of tissue. In doing so one would need to assume a reproducible extraction of enzyme into the supernatant every time during homogenisation. The reproducibility studies performed in this study showed that both the technique adopted in this study and the technique used by Jackson et al, were equally reproducible (Coefficient of variation = 7% for tissue dismembranator, 9% for homogeniser). However with both processes, a small variation was seen in the amount of total protein extracted. Therefore, in the present study the amount of enzyme present in the homogenate was expressed in relation to total protein concentration in an attempt to reduce errors caused by homogenisation. This may account for some of the differences seen by Jackson et al, (1996) but which were of lesser magnitude in the present study.

Matrix metalloproteinase expression plays only a part in the regulation of collagen turnover. As mentioned earlier, the metalloproteinases are inhibited by tissue inhibitors (TIMPs), which are produced locally within the tissues and bind to the MMP enzyme in a 1:1 relationship. An increase in proteinase expression without an increase in the expression of its inhibitor is consistent with an increase in proteolysis and tissue degradation. Therefore, one cannot assume tissue degradation occurs by measuring MMP expression alone. One must also measure TIMP expression.

Experimental models of arthritis have shown tissue destruction occurs in association with an imbalance of metalloproteinase expression over TIMP expression. Synovial explants taken from a rabbit of model arthritis showed increased MMP activity over TIMP synthesis, (Murphy et al, 1993). Studies of osteoarthritic cartilage found considerably elevated levels of MMPs and only moderately elevated levels of TIMP, (Dean et al, 1989). Subsequently, a detailed study of explants taken from patients with osteoarthritis and rheumatoid arthritis has confirmed an excess of MMP expression over TIMP is seen in areas of cartilage degradation (Martel-Pelletier et al, 1994). Cultured smooth muscle cells synthesise less collagen when there is an increase in MMP activity over TIMP activity *in vitro*, (Pickering et al, 1997).

This is the first study to look at both pro and active MMP-2, pro-MMP-9 and TIMP-2 expression in vaginal skin and pelvic fascia in relation to prolapse. As stated earlier, MMP-2 and 9 expression was predominantly higher in women with prolapse compared with controls. TIMP-2 expression was only marginally increased in vaginal biopsies from women with prolapse compared with controls, which was not statistically significant.

When separating the two study groups out into premenopausal and postmenopausal status results for MMP and TIMP expression some interesting results were revealed. There was a trend for MMP-2 and 9 expression to be greater in both premenopausal and postmenopausal women with prolapse, and was statistically significant with regard to pro-MMP-2 ($p<0.05$ for both premenopausal and postmenopausal states). Further to looking

at enzyme expression, the relationship between MMP with TIMP expression was explored.

In both premenopausal and postmenopausal controls, there was a positive relationship between TIMP-2 and both the pro and active forms of MMP-2. Again this positive correlation was found in postmenopausal women with prolapse ($p<0.01$, $R=0.8$ for TIMP-2:pro-MMP-2; $p<0.01$, $R=0.9$). However, there was a negative correlation between MMP-2 and TIMP-2 expression in premenopausal women with prolapse. This may suggest two different mechanisms for the aetiology of prolapse in the pre and postmenopausal groups. The positive relationship between MMP and TIMP expression in both controls and postmenopausal prolapse vaginal tissue may reflect tissue remodeling where the balance of MMP and TIMP activity is in dynamic equilibrium. In premenopausal vaginal tissue the negative correlation between MMP and TIMP expression may reflect a fall in TIMP activity or increase in MMP activity thus causing an imbalance in the equilibrium towards collagen breakdown rather than remodeling. This may in time lead to the development of prolapse.

Approximately 14% of collagen is composed of the amino acid hydroxyproline. Numerous studies have used the measurement of hydroxyproline to estimate the collagen content of tissue samples (Stegeman and Stalder, 1967). Jackson et al (1996), found a reduction in hydroxyproline content in biopsies of vaginal tissue from premenopausal women with prolapse compared with controls. However, there are widely different results in the literature regarding hydroxyproline content and prolapse / genuine stress

incontinence. Keane et al (1997), examined samples of tissue taken from the vesico-vaginal fascia in 36 premenopausal nulliparous women with genuine stress incontinence and found a reduced collagen content in these biopsies compared with controls. However, Sayer et al (1990), took similar biopsies from 60 women with genuine stress incontinence and showed no difference in the hydroxyproline content of tissue from premenopausal women with bladder neck prolapse compared with controls. They did note a difference in the quality of the collagen, which they attributed to changes in cross-links, but not in the quantity of collagen. A recent paper by Falconer[1] et al, (1998) showed a 30% increase in the total collagen content in 15 premenopausal women with incontinence, compared with 16 age matched control. Interestingly, they found by indirect methods, there was an increase in the collagen crosslinks in these tissue biopsies. They suggested that in women with incontinence the connective tissue was more rigid with an impaired mechanical function compared with continent controls. Clearly, there are contradictory studies in the literature about the quantity or quality of collagen and their association with the development of prolapse or genuine stress incontinence. Within this study there was no significant change in the hydroxyproline content of vaginal tissue of both premenopausal and postmenopausal women when comparing women with prolapse with controls without prolapse. However, mean levels of hydroxyproline were lower in the postmenopausal women and significant differences may be apparent with larger numbers, as looking at the relationship between MMP expression and hydroxyproline content, again reveal interesting results.

No statistically significant results were found when assessing the relationship between the expression of different components in vaginal tissue of premenopausal women .In contrast statistically significant relationships were seen in the postmenopausal tissue of women with prolapse. In postmenopausal controls there was no significant relationship between pro and active MMP-2 expression and hydroxyproline content. However, in postmenopausal women with prolapse a significant positive relationship was seen between MMP-2 expression and hydroxyproline content (pro-MMP-2 : TIMP-2 $p<0.01$, $r=0.85$; pro-MMP-2 : hydroxyproline content, $p<0.01$, $r=0.879$; active MMP-2 : TIMP-2, $p<0.01$, $r=0.903$; active MMP-2 : hydroxyproline content, $p<0.05$, $r=0.725$). This suggests that the increase seen in MMP-2 expression is associated with an increase in TIMP-2 expression resulting in an increase rather than decrease in collagen deposition. This again may reflect tissue remodeling. In premenopausal women with prolapse there is no relationship between MMP-2 expression and TIMP-2 expression. If the numbers of patients in the premenopausal group were higher then perhaps a negative relationship may be predicted as seen in the relationship between MMP-2 and TIMP-2 expression. This would explain a different mechanism for the aetiology of prolapse, similar to that suggested by Jackson et al, (1996). Interestingly, this group only studied premenopausal women. Their findings suggesting collagen degradation would support the work in this thesis. Analysis of two papers by Falconer[1&2] et al, (1998) supports the theory that a different collagen metabolism may exist in premenopausal compared with postmenopausal women with stress incontinence. The group examined hydroxyproline content and cross-linking in the paraurethral tissues of women with genuine stress incontinence compared with controls. They found paraurethral tissue from premenopausal women with GSI had 30% more

collagen and higher cross-linking than similar tissue from premenopausal continent controls. This is contrary to Jackson et al's findings. However, in a consecutive publication (Falconer et al, 1998[2]), analysis of postmenopausal patients found no difference in the hydroxyproline content of postmenopausal women with GSI compared with controls. If one looks carefully at both papers the group found tissue from postmenopausal women with GSI had a greater hydroxyproline content than similar tissue from premenopausal women with GSI (Falconer [1], et al, 1998). This would support the theory that there may be two mechanisms for the development of prolapse between the premenopausal population and postmenopausal population.

7.2 Collagen metabolism in the uterosacral ligaments and its relationship with collagen metabolism in the vaginal tissue

This is the first study to examine samples of uterosacral ligament from women with prolapse and controls. Numerous workers have studied vaginal skin biopsies and suggested that similar changes in collagen metabolism occur in the supportive structures. The aim of this study was to determine and quantify any differences in hydroxyproline content, pro and active MMP-2, pro-MMP-9 and TIMP-2 expression in the uterosacral ligaments of women with prolapse and controls, and assess if there was a relationship between the assays from skin and assays from the uterosacral ligament. An assessment of the literature with regard to the anatomy of pelvic support, suggested the uterosacral ligaments were the most important fascial structures involved in holding the uterus and cervix in place. This is from both from cadaveric studies, (Mengert, et al, 1936; DeLancey et al, 1993), as well as clinical reports documenting uterine prolapse following laparoscopic ablation of the uterosacral nerves (Good et al, 1992; Davis 1996). The site of the biopsies taken from the uterosacral ligament was chosen as 1 centimetre from their insertion into the uterus as this was considered on histological analysis to be the portion of the uterosacral ligament with the most structural integrity (Campbell 1950).

Pro MMP-2, active MMP-2 and pro-MMP-9 expression were elevated in the biopsies of uterosacral ligament from both the pre and postmenopausal women with prolapse compared with matched controls. However, in contrast to the vaginal skin biopsies, these

did not reach statistical significance. In addition, there was no significant difference in the hydroxyproline content of the uterosacral ligaments between the two groups.

There was no relationship between pro or active MMP-2 expression and TIMP-2 expression in uterosacral ligament samples from premenopausal and postmenopausal controls. However, there was a significant positive relationship between pro and active MMP-2 expression with TIMP-2 expression in the postmenopausal women with prolapse (relationship pro-MMP-2 and TIMP-2 :$p<0.01$, $r=0.85$; relationship active MMP-2:TIMP-2, $p<0.01$, $r=0.84$). This was associated with a positive relationship between pro and active MMP-2 expression and hydroxyproline content in samples of uterosacral ligament from postmenopausal women with prolapse (pro-MMP-2: hydroxyproline, $p<0.05$, $r=0.69$; active MMP-2: hydroxyproline, $p=0.055$, $r=0.69$). In premenopausal women with prolapse there was a negative trend between pro and active MMP-2 expression and TIMP-2 expression, as well as a non significant negative relationship between MMP-2 expression and hydroxyproline content. These results are similar to those found in vaginal skin. These results suggest that in postmenopausal women with prolapse, a rise in MMP-2 expression was associated with a rise in TIMP-2 expression and hydroxyproline content which may suggest collagen deposition and tissue remodelling rather than degradation. In contrast, in controls and premenopausal women with prolapse, a rise in MMP-2 expression was associated with a fall in TIMP-2 expression and reduction in hydroxyproline content, although this was not significant. The statistical results may reflect a type 2 error and larger numbers may have revealed significant results. The initial power calculation was based on the work by Jackson et al, (1996), however as stated earlier the differences in MMP expression in this study are less pronounced than in the

study by Jackson et al, which may be due to differences in the techniques used for homogenization (see section 7.1). This study corroborates the findings in the premenopausal vaginal tissue, and may suggest a different mechanism for the rise in MMP expression seen in prolapse associated with the postmenopause (tissue remodeling secondary to prolapse) rather than the premenopause (tissue degradation causing prolapse).

Histologically vaginal fascia and the uterosacral ligament are composed of different constituents. The uterosacral ligaments have a high proportion of smooth muscle within their connective tissue (Campbell 1950; own observations) which is absent in the vaginal skin. This would suggest the uterosacral ligaments are a stronger tissue than vaginal skin and provide a different function. Adams et al, have recently shown that the mechanical properties of tissues from different sites around the pelvis are very different (personal communication; Adams et al, 2001). The group studied biopsies taken from the vaginal skin, uterosacral, cardinal and round ligaments of 66 pre and postmenopausal women: 33 women with prolapse and 33 controls. Currently data is only available on the control group. Adams et al, have shown that the load and distension parameters for vaginal skin is significantly different to that of the uterosacral ligaments. The group found no differences in the biomechanical properties with regard to pre and postmenopausal status.

In this study there was no significant difference in the expression of pro-MMP-2, active MMP-2, pro-MMP-9, TIMP-2 and hydroxyproline content in samples of vaginal skin and ligament taken from the same patient. Significant differences in pro-MMP-2 expression

were only seen in the vaginal tissue of women with prolapse and controls. However, no significant differences were seen in the uterosacral ligaments of the same women as the changes in collagen metabolism appeared to be smaller than in the vaginal tissue. These findings may be due to two reasons. Firstly, the study may be too small to show a significant difference in the smaller changes in collagen metabolism in the uterosacral ligaments, but were appropriate to demonstrate a significant difference in the vaginal tissue. Secondly, the exaggerated results seen in the vaginal skin may be due to changes caused by the prolapse, rather than a cause of the prolapse. If the prolapse was solely due to an inherent increase in metalloproteinase expression throughout the body, one would expect to see a similar or greater expression of metalloproteinases in the uterosacral ligaments as they support the uterus and so should be more susceptible to their effects than the vaginal skin. In contrast the vaginal skin is a more elastic structure and is designed to stretch to accommodate structures within the vagina such as the baby during parturition. The significant rise in MMP expression, and concurrent rise in TIMP expression seen in the vaginal skin and not in the uterosacral ligaments, may be the tissue remodeling itself in response to the tissue being stretched. If the rise in MMP and TIMP activity occurs as a result of stretching, it would seem reasonable to expect tissue that has resisted stretching to have a lower MMP expression than those tissues that have not resisted stretching.

There is some evidence in the literature to support this hypothesis. It has been shown that stretching alone can evoke the release of MMP's from tissues *in vitro*. Maradny et al, took strips of fetal membranes and muscle from the lower uterine segment and stretched them in an organ bath. They showed that by stretching the samples of tissue they could

demonstrate a significant increase in collagenase activity (Maradny et al, 1996). Remodeling of the extracellular matrix may be occurring in tissues rather than proteolysis in response to stretching. This would explain the associated rise in TIMP activity alongside the rise in MMP activity as well as there being no overall change in the hydroxyproline content. This would be supported by the work of Sayer et al (1990) and Jackson et al (1996), who commented on changes within the collagen cross-linkages in patients with prolapse, which Jackson suggested was due to "immature" newly laid down collagen which would be seen in tissue remodeling.

Unfortunately the quantification of collagen cross-linkages was not possible using current laboratory means and methods available for this study. Currently the only centre with the expertise to directly quantify collagen cross-linkages in the UK is the Bristol Research Group, from whom technical advice was sought. Workers have looked at cross-linking indirectly. Sayer et al, (1990) found increased autofluoresccence of collagen samples from women with stress incontinence and suggested this was due to cross-linking. Falconer et al, quantified cross-links by measuring the degree of collagen extraction after pepsin digestion of samples (1994). However, this only measures cross-links that are lysed by pepsin digestion, which does not account for all cross-linking. Quantification of collagen cross-linkages would have been useful in this study to determine if different mechanisms of remodeling / degradation occur in the pre and postmenopausal groups. One may hope to find greater immature cross-linkages and static collagen content in tissue that is being remodeled, compared with static cross-linkages and reduced collagen content in tissue that is being degraded.

Chapter 7: DISCUSSION

Previous workers have examined the ratio of collagen types within tissue samples. The main fibrillar collagens are types I and III. Type I collagen forms strong fibres, organised into large bundles, whereas type III collagen is more flexible, with more randomly organised fibres. It had been thought that tissues with a higher ratio of type III to type I collagen are weaker and prone to prolapse. Keane et al, found a decreased ratio of type I to type III collagen in women with stress incontinence (Keane et al, 1997). Jackson et al, however, showed no difference in the ratio of type I:type III collagen in women with prolapse (Jackson et al, 1996). At the start of this study, Western blot analyses were performed on some samples to demonstrate and semi-quantify the presence of collagen type III in homogenised tissue samples. However, discussion with the Bristol Collagen Research Group, (N. Avery) revealed that as the tissues age, the inter-fibrillar bonds become more resistant to cyanogen bromide digestion which is involved in the quantification of collagen types. As women in this study were both premenopausal and postmenopausal, resistance to cyanogen bromide digestion would be variable and make it difficult to comment on collagen type ratios. In view of this the Western blot analyses and quantification of collagen types was abandoned.

7.3 Oestrogen receptor expression and smooth muscle phenotype in the uterosacral ligaments

The prevalence of prolapse increases with age, especially during the postmenopausal period (Mant et al, 1997), suggesting that hypo-oestrogenism may be an important aetiological factor. The presence of the oestrogen receptor is considered to be a prerequisite for determining target organs for the action of oestrogen. Mokrzycki et al, (1997), demonstrated the presence of oestrogen receptors in the uterosacral ligament and suggested that the oestrogen receptor may be a target for treatment of prolapse. However, in their group of 19 premenopausal and 6 postmenopausal women there was only one woman with prolapse. The aim of this study was to determine if there was an association between the quantity of oestrogen receptor expression in the uterosacral ligaments and the presence of prolapse. As oestrogen receptor expression may alter with menopausal status, women were matched for their age, hormonal status for women with prolapse compared with controls.

It was decided to match patients even for age and menopausal status. In addition patients were matched for cycle phase as there is some evidence that oestrogen receptor expression alters during the menstrual cycle. Snijders et al (1992), observed a significant rise in oestrogen receptor expression in endometrial cells during the proliferative phase and fall in the secretory phase. This may have been a predictable result as the composition of the endometrium alters during the cycle in response to changes in oestrogen and progesterone concentration. However, they also noted a change in the oestrogen receptor expression in

the smooth muscle of myometrium during the cycle. Changes in oestrogen receptor expression with phase of the menstrual cycle, have been documented in other tissues within the pelvis including the cervical myometrium and ectocervical epithelium (Punnonen & Lukola, 1982). Sjoberg et al (1989), showed similar fluctuations in oestrogen receptor expression in the vaginal epithelium during the menstrual cycle, however, this was not supported by Di Carlo et al (1985), who found no change in oestrogen receptor concentration within the vaginal epithelium, either during the menstrual cycle or after the menopause. There is no literature available regarding fluctuations in oestrogen receptor expression in the supportive pelvic tissues. However, in view of the evidence regarding the endometrium, myometrium, cervix and vagina, it was felt oestrogen receptor expression may also alter with cycle phase and so patients were matched for cycle phase and hormonal status to exclude any bias introduced by their hormonal status.

The results of this study confirm the findings of Mokrzycki et al (1997). In histological sections of the uterosacral ligament, the oestrogen receptor was confined to the nuclei of the smooth muscle cells of the uterosacral ligaments. There was no staining found in the smooth muscle cell nuclei of blood vessels or any other cell type. A similar staining pattern of smooth muscle cells with vascular smooth muscle sparing, is seen within the myometrium (Press et al, 1984). However, the oestrogen receptor does not only exist in smooth muscle. The oestrogen receptor has been demonstrated in fibroblasts as well as smooth muscle cells in the vagina (Hodgins et al, 1998). Quantification of the oestrogen receptor showed no difference in the quantity of oestrogen receptor expression between

women with prolapse compared with controls. In addition, there did not appear to be any alteration in oestrogen receptor expression in controls or women with prolapse after the menopause, which supports the work by Mokrzycki (1997). There was no correlation between hydroxyproline content and oestrogen receptor expression. The study population was too small to comment on oestrogen receptor expression and cycle phase.

This study demonstrates there appears to be little relationship between oestrogen receptor content and prolapse. However, the presence of the oestrogen receptor within the smooth muscle of the uterosacral ligaments suggests there they may be a target for treatment of prolapse. Although no study has examined the effect of oestrogen supplementation on established prolapse there is scanty evidence that hormone replacement has little/no effect in treating genuine stress incontinence, which is thought to occur due to decreased support of the bladder neck. Karram et al (1989), performed urodynamics on 6 postmenopausal women before and after oestrogen supplementation and found no change in urethral pressure, functional length or cystometry after oestrogen administration. Jackson et al, (2002) recruited women with genuine stress incontinence to a double, blind, randomised, placebo controlled trial of oestrogen valerate 2mg once daily for 6 months. After this period of time they found no significant benefit of using oestrogen supplementation for the treatment of postmenopausal genuine stress incontinence.

However, some studies have shown an effect of oestrogen administration on urodynamic parameters and the pelvic connective tissue. Hilton et al, showed an increase in the maximum urethral closure pressure after administration of vaginal oestrogens (Hilton et

al, 1983) and suggested this may be due to changes brought about by impaired collagen. Versi et al, (1988) showed a correlation between urethral pressure profiles and skin collagen content. Falconer[1&2] et al, (1998) found the paraurethral connective tissue behaved differently in postmenopausal women on hormone replacement compared with those not taking oestrogen supplementation.

The existence of different types of oestrogen receptor may explain why although there was no demonstrable difference in the expression of the α receptor with age or prolapse yet oestrogen may still affect the composition and character of the endopelvic fascia. The presence of different oestrogen receptors explains the so called "anti-oestrogenic" effects of tamoxifen on the breast yet unpredictable and often oestrogenic effects on the endometrium and positive effects on the skeleton (Purdie , 1997). A group of drugs classed as "Selective Oestrogen Receptor Modulators" (SERM's) act via different receptors. The best known SERM, Raloxifene, has no oestrogenic effect on the endometrium but acts like an oestrogen by stimulating oestrogen receptors in bone (Purdie, 1997). Recently, Vardy et al (2000), reported that raloxifene and tamoxifen both caused a trend toward increased pelvic organ prolapse after short term treatment and suggested this increased trend was modulated via the oestrogen receptor. Histologically another phenotype of the oestrogen receptor (oestroegn receptor-beta) has been identified which would not have been immunostained using the ID-5 monoclonal antibody to the oestrogen receptor-alpha. Further work is therefore needed to see if the beta-oestrogen receptor is present on smooth muscle cells of the uterosacral ligament, or other cell types that may be involved in the phenomenon described by Vardy et al (2000). In addition,

further work could utilize molecular biology to look at the messenger RNA coding for different oestrogen receptors which occurs upstream from the histological analysis of receptor expression.

The differentiation of smooth muscle cells to myofibroblasts has been well documented. Previous workers have shown cultured smooth muscle cells can undergo a differentiation from a smooth muscle, to myofibroblast form (Ricciardelli et al, 1989, Kocher et al, 1991; Ivarsson et al, 1996). In doing so, the smooth muscle cells change their phenotype from a high desmin and α-smooth muscle actin expression to have lower desmin expression and higher vimentin expression.

Ricciardelli (1989) found cultured explants of prostrate smooth muscle cells changed to myofibroblasts *in vitro*, under the effect of oestrogen. Therefore, the one theory was that the uterosacral ligaments of women with prolapse may have a higher expression of myofibroblasts compared with controls, which would produce increased amounts of matrix metalloproteinases leading to an increase in collagen degradation and prolapse.

Within sections of uterosacral ligament all three phenotypes were demonstrated. There was a high density of staining of α-smooth muscle actin in both the uterosacral ligament smooth muscle, and the smooth muscle around blood vessels. Desmin was predominantly found in the uterosacral ligament smooth muscle rather than the smooth muscle in the walls of surrounding blood vessels, but desmin expression was less pronounced than α-smooth muscle actin. In contrast to the dense positive staining for desmin and α-smooth

muscle actin, vimentin was present in the smooth muscle of the uterosacral ligaments but to a lesser extent compared with desmin and -smooth muscle actin. In contrast vimentin expression was found to be greater in the smooth muscle of blood vessels. The proportions of all three phenotypes were measured to see if different proportions of each phenotype suggested cells had adopted a smooth muscle or myofibroblast lineage. No significant difference in the density of staining for each antigen, was demonstrated in the smooth muscle cells of the uterosacral ligaments between the women with prolapse compared with controls.

A similar distribution and intensity of staining was found by Eyden et al (1992), in the smooth muscle of normal myometrium and leiomyoma. The highly positive staining for desmin and α-smooth muscle actin, suggests the uterosacral ligament smooth muscle cells have a "smooth muscle differentiation" rather than "myofibroblast differentiation" (Santini et al, 1995). The findings of this study suggest the smooth muscle within the uterosacral ligaments appear to retain their capabilities, rather then develop a myofibroblastic phenotype.

Concluding remarks and future studies

This study demonstrates there is an elevation in matrix metalloproteinases in the vaginal tissue of women with prolapse. This supports previous work in the literature (Jackson et al, 1996). Similar trends were seen in the uterosacral ligaments of women with prolapse but these did not reach statistical significance. However, a significant correlation was seen between the expression of pro-MMP-2, active MMP-2, pro-MMP-9 and TIMP-2 in the vaginal tissue and uterosacral ligaments. This suggests that changes in collagen metabolism occurring within the uterosacral ligaments of women with prolapse are reflected in the vaginal skin. The alterations in collagen metabolism are more exaggerated in the vaginal tissue and may reflect a combination of both inherent elevation in MMP expression as well as a response to tissue stretching. In addition findings may suggest a different mechanism for prolapse in the postmenopausal women compared with the premenopausal women. Prolapse before the menopause appears to be associated with an over expression of metalloproteinase activity in relation to TIMP expression, which may promote the degradation of collagen in the endopelvic fascia. However, after the menopause an elevation in MMP expression is associated with an elevation in TIMP expression, which suggests there is an attempt in the tissue to remodel rather than degrade. These phenomena may occur as a result of the prolapse, rather than a cause. Further studies are needed to see if the changes in collagen metabolism are the cause of prolapse or an effect of stretching? Such studies would include the analysis of vaginal tissue taken from patients with prolapse in different compartments. If samples were taken from the same patient in areas of the vagina where there is distension of the vaginal tissues in

association with prolapse (eg cystocele) and areas where there is no stretching of the vaginal tissue (eg lateral fornices / posterior wall of vagina) then similar tissue types could be examined from the same patient and compared. This would differ from this study where vaginal tissue was compared with ligament in the same patients. If a difference was observed in different vaginal sites, the rise in MMP expression is probably the result of stretching, as inherent abnormalities in MMP expression and collagen metabolism would be present in both sites of biopsies, regardless of whether there is prolapse or not. More work could investigate the presence of messenger RNA for MMP's and other enzymes implicated in tissue degradation. This would demonstrate that the signal for production of MMP's was still present and factors influencing the signal could be investigated.

Oestrogen receptor expression was seen in the nuclei of smooth muscle cells in the uterosacral ligaments, but no difference in expression was seen between prolapse compared with controls. The presence of the oestrogen receptor suggests it may be a target for the treatment of prolapse, so further work is needed to determine if administration of oestrogen may affect collagen metabolism or whether the beta-oestrogen receptor is present in the smooth muscle of the uterosacral ligaments and if differences occur in expression in women with prolapse.

In conclusion, this study has shown that collagen metabolism in the vaginal tissue is a reflection of that in the uterosacral ligaments, and that oestrogen administration could target the uterosacral ligaments.

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APPENICES 1: Patient details: age parity, ICS stage

	stage prolapse	age	parity		Stage Prolapse	age	parity
PROL1	3	60	5	**CON1**	0	63	2
PROL2	3	64	5	**CON2**	0	67	3
PROL3	3	74	4	**CON3**	0	74	2
PROL4	3	53	1	**CON4**	0	51	0
PROL5	2	87	2	**CON5**	0	82	1
PROL6	3	62	7	**CON6**	0	74	1
PROL7	2	55	1	**CON7**	0	56	2
PROL8	2	74	2	**CON8**	0	84	0
PROL9	2	38	3	**CON9**	1	36	4
PROL10	2	45	3	**CON10**	0	40	2
PROL11	2	42	3	**CON11**	0	43	2
PROL12	2	48	2	**CON12**	1	42	3
PROL13	3	51	4	**CON13**	0	41	4
PROL14	3	46	5	**CON14**	0	48	6

APPENICES 2: Collagen related assays in prolapse vaginal tissue

	pmmp2/[prot] Skin au/mg	ammp2/[prot] Skin au/mg	pmmp9/ [prot] Skin au/mg	Timp2/[prot] Skin ng/mg	Hproline Skin umol/g
PROL1	0.051229508	0.025614754	0.014344262	0.5	54.02844167
PROL2	0.167890871	0.054564533	0.012591815	18.18	104.6860265
PROL3	0.110619469	0.1179941	0.022123894	15.67	77.74154286
PROL4	0.084269663	0.146067416	0.016853933	22.6	102.5685366
PROL5	0.052917232	0.030529172	0.044775512	1.86	57.54966851
PROL6	0.075614367	0.058601134	0.054545455	4.95	69.31905036
PROL7	0.209090909	0.172727273	0.022916667	33.77	145.4293889
PROL8	0.104166667	0.054166667	0.025782689	10.26	107.3457468
PROL9	0.033149171	0.018416206	0.071743929	7.28	176.09195
PROL10	0.107615894	0.049668874	0.024911032	3.14	96.35858537
PROL11	0.03202847	0.010676157	0.035447761	7.44	66.85126429
PROL12	0.052238806	0.042910448	0.041450777	2.22	127.4281188
PROL13	0.088082902	0.051813472	0.028725314	5.64	95.15372727
PROL14					65.14952747

APPENICES 3: Collagen related assays in control vaginal tissue

	pmmp2/[prot] Skin au/mg	ammp2/[prot] Skin au/mg	pmmp9/ [prot] Skin au/mg	timp2/[prot] Skin Ng/mg	Hproline Skin umol/g
CON1	0.029411765	0.015837104	0.012669683	11.5	83.64285714
CON2	0.114169215	0.032619776	0.011132873	1.46	115.7189381
CON3	0.059674503	0.059674503	0.059675582	2.51	80.96415385
CON4	0.036511156	0.081135903	0.015415822	11.6	203.6562637
CON5	0.026041667	0.044642857	0.042410714	1.93	264.9883099
CON6	0.015018773	0.015018773	0.013516896	0.58	262.1247761
CON7	0.026315789	0.039473684	0.015350877	0.93	50.93817293
CON8	0.129268293	0.065853659	0.03902439	33.1	97.52462264
CON9	0.013986014	0.018648019	0.018648019	9.55	94.54432258
CON10	0.092009685	0.03874092	0.016949153	10.2	89.91664122
CON11	0.01242236	0.016563147	0.009937888	6.46	119.53928
CON12	0.040785498	0.056646526	0.058911497	16.8	77.95659898
CON13	0.03196347	0.02739726	0.017351598	1.84	62.16091379
CON14					144.3492464

APPENICES 4: Collagen related assays in prolapse uterosacral ligaments

	pmmp2/[prot] Lig au/mg	ammp2/[prot] Lig au/mg	pmmp9/ [prot] Lig au/mg	timp2/[prot] Lig ng/mg	Hproline Lig umol/g
PROL1	0.054852321	0.037974684	0.012658228	7.07	64.15601266
PROL2	0.177187154	0.062015504	0.022123894	10.14	92.29091429
PROL3	0.097328244	0.097328244	0.016	8.64	67.12162097
PROL4	0.065522621	0.065522621	0.031824777	7.99	54.7784539
PROL5	0.052132701	0.09478673	0.037914692	8.84	72.88297436
PROL6	0.025210084	0.06302521	0.01092437	3.43	79.72422222
PROL7	0.219298246	0.14619883	0.065789474	30.13	109.0232727
PROL8	0.055335968	0.035573123	0.013833992	4.13	52.81745294
PROL9	0.09103079	0.037483266	0.053547523	5.37	69.3716
PROL10	0.071496663	0.095328885	0.038131554	6.49	75.28974359
PROL11	0.017182131	0.005154639	0.010652921	12.87	132.0405785
PROL12	0.056140351	0.060818713	0.028070175	1.17	111.364375
PROL13	0.041493776	0.008298755	0.014107884	2.65	35.85269663
PROL14					78.2087629

APPENICES 5: Collagen related assays in control uterosacral ligaments

	pmmp2/[prot]	ammp2/[prot]	Pmmp9/ [prot]	timp2/[prot]	Hproline
	Lig	Lig	Lig	Lig	Lig
	au/mg	au/mg	au/mg	ng/mg	umol/g
CON1	0.035714286	0.017857143	0.016071429	6.78	69.78142105
CON2	0.069387755	0.016326531	0.016326531	1.38	93.29994118
CON3	0.054054054	0.058558559	0.047297297	2.13	79.8960989
CON4	0.03611898	0.074362606	0.015299618	5.84	121.9326809
CON5	0.03902439	0.043902439	0.024390244	1.2	104.0945695
CON6	0.04400978	0.068459658	0.014180929	0.73	81.71511111
CON7	0.066945607	0.050209205	0.012552301	4.23	78.8379375
CON8	0.107142857	0.081632653	0.030612245	4.15	35.7157697
CON9	0.048387097	0.032258065	0.026209677	5.43	91.86595376
CON10	0.0792	0.048	0.028800461	6.93	110.5761417
CON11	0.013565891	0.011627907	0.012403101	1.23	76.03923529
CON12	0.065530799	0.070773263	0.062909567	6.72	95.24096774
CON13	0.035714286	0.011160714	0.008035714	8.7	62.48756701
CON14					87.2419731

APPENDICES 6: Oestrogen receptor expression and smooth muscle phenotype in prolapse uterosacral ligaments

	e2rec % +ve nuclei	vimentin % stained	desmin % stained	sm actin % stained
PROL1	30	4	21	63
PROL2	66	2	31	46
PROL3	71	3	57	93
PROL4	94	3	35	69
PROL5	87	9	60	96
PROL6	70	5	37	83
PROL7	82	2	31	79
PROL8	73	1	13	64
PROL9	92	4	35	74
PROL10	33	1	38	29
PROL11	69	6	38	83
PROL12	70	1	25	34
PROL13	88	5	61	55
PROL14	89	3	39	55

APPENDICES 7: Oestrogen receptor expression and smooth muscle phenotype in control uterosacral ligaments

	e2rec % +ve nuclei	vimentin % stained	desmin % stained	sm actin % stained
CON1	18	1	11	56
CON2	60	10	35	55
CON3	86	5	57	93
CON4	86	1.1	50	74
CON5	68	5	45	92
CON6	55	1	28	62
CON7	54	3	58	53
CON8	82	1	36	67
CON9	79	4	39	68
CON10	65	2	13	51
CON11	31	6	29	74
CON12	63	2	15	76
CON13	79	5	41	65
CON14	86	4	24	66