Cellular and Molecular Characterisation of Pelvic Ligaments Fibroblasts: Effects of Gonadal Steroids

Thesis submitted for the degree of

Doctor of Medicine

Department of Cancer Studies and Molecular Medicine
Leicester and Warwick Medical School
University of Leicester

2006

By

Ayman A. A. Ewies

MBChB, MSc, MRCOG
To my parents, brother and sister

To my wife and three children
High Zeal

The one who has dignity must frown upon any deficiency that can be eliminated from his soul. For if prophet-hood was gained through effort, then a person would be regarded as deficient if he was pleased with monarchy.

Ibn Al-Jawzey
1116-1200
Acknowledgements

My foremost thanks go to my supervisor Mr. Farook Al-Azzawi, for his support, help, advice, encouragement in pursuing this research, and for creating an atmosphere of productivity within the department.

I am grateful to Professor John Thompson, for devoting a great deal of his time to help with the statistical analysis, and for his patience in discussing the results.

I am indebted to Dr. May Wahab, for her advice as well as patience and determined effort to teach me tissue culture and immunohistochemical techniques.

I am particularly grateful to Dr. Mona El-Shafie, for her invaluable contribution in the assessment of cytoskeleton morphology.

My thanks go also to Dr. Jerry Styles, for his hands-on help in cDNA microarray experiments.

I would like to thank Dr. Adrian Stanley and Dr. Jin Lee for their help in the stretch experiments and RNA extraction, respectively.

Last not least, my thanks go to the Department of Obstetrics and Gynaecology. I would like to thank Professor David Taylor, Head of the Department, for his encouragement and support. In addition, all the technical staff deserves my thanks, particularly Mrs. Juliet Jackson, for ensuring that technical and administrative matters ran smoothly.
The laboratory work in the thesis

1- **Immunohistochemistry**: I have developed and optimised the protocols for the immunohistochemical staining of extracellular matrix proteins. I have performed all the staining for both steroid receptors and extracellular matrix proteins.

2- **Image Analysis**: I have done the capture and subsequent morphometric analysis of all images described in this thesis.

3- **Primary fibroblast culture**: I have designed, optimised and performed all the experiments mentioned in this thesis.

4- **Stretch experiments**: I have designed, optimised and performed all the stretch experiments with initial technical help from the staff of the Department of Medicine.

5- **Fluroprobe experiments**: I have designed, optimised and performed all the experiments described in this thesis. I have assessed the actin morphology jointly with the Gynaecology Research Group’s morphologist.

6- **RNA extraction**: I have performed the experiments mentioned in this thesis. The quality and integrity of RNA were assessed by the Gynaecology Research Group’s scientist using Agilent Bio-analyser.

7- **cDNA microarray experiments**: These were performed by the team at the MRC Toxicology Unit, Leicester University under the supervision of Dr. Tim Gant.
Abstract

The high incidence of pelvic organ prolapse (POP) observed in postmenopausal women and in those used levormeloxifene suggested an aetiological role for the hypoestrogenic state. Further, the oestrogen-related molecular and structural changes in hip and knee ligaments, regulation of oestrogen action by extracellular matrix (ECM) proteins, and the high expression of steroid receptors in the mechanically failed connective tissue were previously reported. These observations led to the hypotheses of changed ECM composition with compensatory increase in steroid receptors expression in the prolapsed cardinal ligaments. A series of immunohistochemical studies proved the assumption that the prolapsed cardinal ligaments would have higher expression of collagen III (p=0.001), tenascin (p=0.001), oestrogen receptor α (p=0.09), androgen receptor (p=0.004), progesterone receptor (p=0.03), and lower expression of elastin (p=0.004) when compared to non-prolapsed ligaments, irrespective of menopausal status. Unpredictably, collagen I expression was directly related to the menopausal status rather than prolapse, and the prolapsed ligaments showed lower expression of oestrogen receptor β (p=0.02). HRT was assumed to rectify the prolapse-related changes in postmenopausal women, but it had only partial ameliorating effect by reducing collagen III (p=0.001) and androgen receptor (p=0.06) expression in the prolapsed ligaments to levels similar to the normal ones.

Given the immunohistochemical findings, it was hypothesised that a number of genes representing the altered ECM proteins and gonadal steroids receptors would be modified. The stretch model was used assuming that the effect of long duration mechanical stretch on primary cultures of fibroblasts derived from cardinal ligaments in vitro would be similar to the effect of chronic stretch associated with prolapse in vivo. cDNA microarray identified genes coding for regulation of actin remodelling, but it fell short of identifying alterations in gene expression commensurate with the findings of immunohistochemistry. This might be attributed to the differences between the in vivo and in vitro environment and/or the design of the stretch model used in this experiment.

The cDNA microarray findings led to the hypothesis that mechanical strain e.g. in cases of increased intra-abdominal pressure and levormeloxifene could cause POP by destroying the cytoskeleton of the fibroblasts. To test this hypothesis, the effect of mechanical stretch and levormeloxifene on the morphology of the cytoskeleton of those fibroblasts grown in primary cultures was studied using fluorescein technology. The effect of 17β-oestradiol was also investigated assuming that it would prevent and/or reverse the effect of levormeloxifene and stretch. Stretch caused major phenotypic alterations in actin morphology (p=0.0001), and levormeloxifene caused similar changes in the static fibroblasts (p=0.0001). Nonetheless, the use of oestradiol did not protect the cytoskeleton, but significantly increased cell proliferation (p=0.02), which was reduced by stretch (p=0.001), suggesting a beneficial role in the healing process.

By submitting this thesis and the publication of this set of articles, it is hoped that the area of prolapse will be opened up for further objective assessment of cell-matrix interactions, and additional opportunities for creative exploration will be catalysed.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF</td>
<td>Actin depolymerising factor</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin-related protein</td>
</tr>
<tr>
<td>CI/CIII</td>
<td>Collagen I / Collagen III</td>
</tr>
<tr>
<td>DNase 1-L1</td>
<td>Deoxyribonuclease 1-like 1</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidyl inositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PIP5K1C</td>
<td>Phosphatidyl inositol-4-phosphate 5-kinase</td>
</tr>
<tr>
<td>POP</td>
<td>Pelvic organ prolapse</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective oestrogen receptor modulators</td>
</tr>
<tr>
<td>SIPA-1</td>
<td>Signal-induced proliferation associated gene-1</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Transforming growth factor-β3</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TN-C</td>
<td>Tenascin-cytotactin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFRSF1A-associated via death domain</td>
</tr>
<tr>
<td>WASP</td>
<td>Wilcott-Aldrich Syndrome Protein</td>
</tr>
</tbody>
</table>
# Table of Contents

**Chapter 1: Introduction**
- Historical background .......................................................... 1
- Pelvic organ prolapse in modern gynaecology .......................... 2
- Contributing factors to pelvic organ prolapse ......................... 4
- Stages of pelvic organ prolapse .................................................. 6
- Structure of ligaments ............................................................ 7
  - Histological structure ....................................................... 8
  - Biochemical composition .................................................. 11
- Extracellular matrix ............................................................. 12
  - Composition and function ................................................. 12
  - Cell adhesion to extracellular matrix ................................. 15
  - Regulation of extracellular matrix ..................................... 16
  - Extracellular matrix and pelvic floor disorders .................... 17
- Cytoskeleton ............................................................................ 18
- Extracellular matrix-actin interaction .................................... 19
- Sex steroid receptors .............................................................. 21
  - Oestrogen Receptor ......................................................... 21
  - Progesterone receptor ...................................................... 25
  - Androgen receptor .......................................................... 27
  - Role of oestrogen in pelvic floor function ............................ 28
  - Levormeloxifene and pelvic organ prolapse ......................... 29
- Mechanical stretch ............................................................... 29
- Microarray technology .......................................................... 30
  - Background .......................................................................... 30
  - Technology overview ........................................................ 31
  - Design issues ....................................................................... 34
  - Variability and replications ............................................... 35
  - Data analysis strategies ..................................................... 37

**Chapter 2: Aims of the research programme** .......................... 39

**Chapter 3: Materials and methods** ...................................... 44
- Immunohistochemical studies ............................................... 45
- Primary culture of fibroblasts ................................................ 60
- Microarray experiments ........................................................ 65
- Cytoskeletal proteins studies ................................................. 77
- Optimisation experiments ...................................................... 80
- Background of the statistical analysis ................................... 81

**Chapter 4: Changes in the extracellular matrix proteins expression** 84
- Introduction ........................................................................... 85
- Aim of the study .................................................................... 86
- Materials and methods .......................................................... 86
Chapter One

Introduction
For many years a neglected subject, pelvic organ prolapse is now attracting increasing interest as a fruitful area for research. Further understanding will come from basic scientific research in conjunction with morphological studies, and advances in imaging should enable this. As our understanding of the pathophysiology of this condition increases, we can then explore preventative measures.

**Historical Background**

The history of pelvic organ prolapse (POP) and proposals for its treatment can be traced past the days of Hippocratic medicine to a record in the Kahun papyrus of Egypt 2000 years before the birth of the Christ. Cleopatra proposed the application of astringent lotions to the vagina in cases of vaginal prolapse. Hippocrates used succussion, in which the patient was suspended upside-down from a ladder and shaken, for the treatment of irreducible uterine prolapse. He suggested that wet feet, excessive exertion, fatigue, and sexual excesses are the aetiological factors of the prolapse. From the days of Hippocratic medicine to the mid-fourteenth century, blocking of the vaginal canal by mechanical means, such as a sponge pack was the most widely accepted method of treatment. Surgical interference was suggested only in cases of gangrenous uterus, and Soranus (around 300 AD) stated unequivocally that it could be done without endangering the patient’s life. The first description of the ligaments of the uterus, “like the sails of a boat”, presumably the broad ligaments, is accredited to Areteus around 300 AD, who stated that uterine prolapse was due to relaxation of these structures.
The first physician to use the word “procidentia” in describing POP was A. Benedetti in 1497, and the first illustration of uterine prolapse, which truly represented the organs involved, was published in 1691 by van Ruysch. The first accurate description of a cystocele, enterocele, and rectocele was by J. Méry in 1713, R. de Garengeot in 1736, and F. Wachter in 1745, respectively. By the end of the 18th century, the terminology which is in use today was established; uterine prolapse of various degrees, relaxation of the anterior vaginal wall or cystocele, relaxation of the posterior vaginal wall or rectocele, true pelvic hernia or enterocele, total prolapse of all pelvic organs or procidentia.

The first vaginal hysterectomy was done by J. Berengario da Carpi in 1521 by placing stout twine around the prolapsed uterus, which was gradually tightened over a period of days until the organ was severed. The stump was treated with a mixture of wine, honey and aloes. Williams Fabry of Hilden described in 1592 a pregnancy in a prolapsed uterus and also a case of uterine prolapse in a virgin. In 1603, R. de Castro suggested attacking the prolapsed uterus with a piece of red-hot iron, as if to burn it, whereupon fright will force the prolapsed part to recede into the vagina. In the 19th century, many operations for the correction of POP were developed, hundreds of different intravaginal pessaries were used and many other modes of treatment were proposed, including the use of cold water hip baths, continuous stream of cold water directed into the vagina, and massage. Choppin of New Orleans, USA performed the first modern vaginal hysterectomy in the world in 1861, and A. Donald of Manchester, England developed the Manchester repair operation in 1888.
From the 16th to the 19th centuries various mechanisms for uterovaginal support were proposed, including vaginal rigidity, perineal support, and the broad and round ligaments of the uterus. It was not until the 20th century that the role of the uterosacral-cardinal ligament complex in supporting the uterus was recognised by Donald and Fothergill. In the early 20th century, the development in anaesthesia and surgical techniques, the adaptation and improvement of suture materials, the invention of surgical equipments and the increased anatomic knowledge led to the development of more sophisticated surgical procedures for the correction of POP. Most of these are essentially modifications of existing techniques, which were incomplete or had serious faults. Nevertheless, the progress in surgical techniques over the past 100 years was not accompanied by satisfactory advances in our understanding of the mechanism of normal pelvic floor function and the aetiology of POP.

**POP in Modern Gynaecology**

POP is one of the greatest morbidities influencing the quality of life, and it is common in both developed and developing countries. It begins during reproductive life and extends potentially through three-quarters of women’s life without much hope of relief unless surgical repair is successful. Surgical techniques for the management of prolapse vary and, in the absence of randomised controlled trials, it remains unknown which options offer the best chance of satisfactory outcome. Estimating the true incidence of POP is difficult because many women accept it as an inevitable consequence of childbirth and ageing. One study reported that over half of women aged
75 years thought that their symptoms were normal for elderly people.\textsuperscript{6} Others are reluctant to come forward through fear or embarrassment, although fortunately attitudes are changing.\textsuperscript{7} While some degree of pelvic floor laxity is present in more than 50\% of parous women,\textsuperscript{8} only 10-20\% of them are symptomatic.\textsuperscript{9} In a report from the Province of Quebec in Canada, POP was accounted for 13\% of all hysterectomies in all age groups.\textsuperscript{10} The woman’s life-time risk for undergoing POP reconstructive or anti-incontinence surgical procedures was calculated to be 11\% by the time she reaches 80 years of age, 30\% of these women had had more than one operation to correct the prolapse.\textsuperscript{11} The incidence of vaginal vault prolapse requiring surgical correction following hysterectomy was 3.6 per 1000 person-years of risk in a cohort of women from family planning clinics followed-up for 26 years. The cumulative risk rose from 1\% three years after a hysterectomy to 5\% after 15 years. The risk of prolapse following hysterectomy was 5.5 times higher in women whose initial hysterectomy was for prolapse rather than for other reasons.\textsuperscript{12} The incidence of POP increases with age. In a series of 190 women with vaginal vault prolapse, it was found that 60\% were over 60 years of age.\textsuperscript{13} Of those awaiting major gynaecological surgery, 20\% had prolapse and this raised to 59\% amongst elderly women.\textsuperscript{7} Despite the rapid increase in the proportion of elderly people in the British society,\textsuperscript{14} which imposes a substantial personal, social and economic burden on the health services in terms of morbidity associated with pelvic floor changes, our knowledge of the effects of aging on the mechanism of pelvic support in human is surprisingly limited.
The mechanism of normal pelvic floor function and POP is poorly understood. It is generally believed that POP has multifactorial aetiology, but it is still an enigma that some women develop prolapse while others with similar risk factors do not. The principal support of the uterus arises from the dynamic actions of the levator ani muscle and the uterosacral-cardinal ligament complex. This ligament complex fixes the upper vagina, cervix and uterus in a posterior direction over the levator plate of the pelvic diaphragm, thus enabling this pelvic valve to counteract increases in intra-abdominal pressure. Moreover, in a normal woman, when standing, the levator plate is nearly horizontal, thus providing support for the pelvic organs. This supportive function of the normal pelvic floor relieves the pressure on the pelvic ligaments. If this function is impaired, the daily tension on these ligaments is increased, leading to continuous deterioration in pelvic floor function over the years, which may become symptomatic if exacerbated by one or more other factors. Recognised factors that contribute to POP are pelvic floor injuries related to pregnancy and childbirth, chronic increase in intra-abdominal pressure, such as heavy lifting and constipation, neurological injury, connective tissue disorders, age-related muscle dysfunction, and hypoestrogenic state in the postmenopausal period. Nonetheless, the occurrence of prolapse in virgins and nulliparas was reported. A single case report in the literature tells of a nulliparous woman experiencing third degree uterine prolapse after a road traffic accident. It was speculated that a sudden increase in intra-abdominal pressure, related to the position of the seat belt, led to the condition. Uterosacral nerve ablation,
during which the uterosacral ligaments are transacted, also was identified as a probable risk factor, underscoring the importance of these ligaments in pelvic support.\textsuperscript{27,28}

**Stages of POP**

There are a number of classifications or grading systems for POP. Those most frequently used are simple, but lack accuracy for scientific comparison. Traditionally, a uterine descent into the upper part of the vagina is called \textit{first degree}. If the prolapse is through the vagina to the region of the introitus, it is \textit{second degree}. If the cervix and uterus prolapse out through the introitus, it is called \textit{third degree, complete or total}.\textsuperscript{29} In 1996, a standardised terminology for the description of female POP and pelvic floor dysfunction was adapted by the International Continence Society, the American Urogynecology Society, and the Society of Gynecologic Surgeons. The terminology replaces such terms as cystocoele, rectocoele, enterocoele and urethrovesical junctions with precise descriptions relating to specific anatomic landmarks.\textsuperscript{30} This prolapse scoring system – pelvic organ prolapse quantification (POPQ) – was shown to be reproducible.\textsuperscript{31} The position of the patient should be noted when assessing prolapse, although a study using this classification system suggested that there is no difference in the degree of POP when the patient is in the dorsal lithotomy position, performing maximum valsalva, or in the standing position.\textsuperscript{32} The first points are on the anterior vaginal wall and categorise anterior vaginal wall prolapse accordingly. Point Aa is a point located in the midline of the anterior wall 3 cm proximal to the urethral meatus and is roughly the location of the urethrovesical crease. Point Ba represents the most
distal position of any part of the anterior vaginal wall. Point C represents either the most distal edge of the cervix or the leading edge of the vagina if a hysterectomy has been performed. Point D represents the location of the posterior fornix (Pouch of Douglas) in a woman with a cervix. Point Bp is a point most distal of any part of the upper posterior vaginal wall and point Ap is a point located in the midline of the posterior vaginal wall 3 cm proximal to the hymen. To record measurements, these points should be expressed in cm above or below the hymen. When the examination is recorded according to the anatomic points just cited, staging may be performed. This is an objective and site-specific system for describing, quantification, and staging pelvic support. It was developed to allow more accurate transmission of information and to make it possible to standardise research information.30 (Table 1.1; Figure 1.1)

**Structure of Ligaments**

**Histological structure:**

Ligaments are pieces of dense connective tissue dominated by collagen fibres that give them a high tensile strength.33 Collagen fibrils, visible ultra-structurally, are grouped into fibres that can be seen by light microscopy. In turn, the fibres are collected into fibre bundles and the bundles into fascicles. The collections of fascicles that form the whole ligament are wrapped up in a surface connective tissue layer called the epiligament.34 A striking feature of ligaments is “crimp”, a regular sinusoidal wave pattern of collagen fibres seen in longitudinal section, that provides a buffer allowing ligaments to elongate without being damaged, and irreversible damage is believed to
**Table 1.1:** Staging of Pelvic Floor Prolapse Using International Continence Society Terminology\(^{30}\)

<table>
<thead>
<tr>
<th>Stage 0</th>
<th>No prolapse is demonstrated. Points Aa, Ap, Ba and Bp are all at -3 cm and either point C or D is between total vaginal length -2 cm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Criteria for stage 0 are not met, but the most distal portion of the prolapse is &gt; 1 cm above the level of the hymen.</td>
</tr>
<tr>
<td>Stage II</td>
<td>The most distal portion of the prolapse is less or equal to 1 cm proximal or distal to the plane of the hymen.</td>
</tr>
<tr>
<td>Stage III</td>
<td>The most distal portion of the prolapse is &gt; 1 cm below the plane of the hymen, but protrudes no further than 2 cm less than the total vaginal length in centimetres.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Essentially complete eversion of the total length of the lower genital tract</td>
</tr>
</tbody>
</table>
Figure 1.1.

Staging of POP
occur only when normal limits of crimp are exceeded. The majority of cells in ligaments are fibroblasts, but myofibroblasts are also present at sites of healing. Fibroblasts have an elaborate shape. They lie in longitudinal rows and have numerous sheet-like cell processes that extend into the extracellular matrix (ECM). The processes surround bundles of collagen fibres and get into contact with processes from cells in adjacent rows. Cells within the same row and those in adjacent rows are linked to each other by gap junctions.

**Biochemical Composition:**

Collagen forms 70-80% of the dry weight of ligaments. This is considered relatively inert metabolically with a half-life of 300-500 days. Most is type I collagen, the principal tensile-resistant fibre, but smaller quantities of type III, V and VI are also present. The proper function of ligaments depends on the appropriate type, synthesis, assembly, cross-linking and remodelling of collagen. The complex interplay between synthesis and remodelling of collagen is influenced by hormones, exercise and immobilisation. The amount of collagen bundles and the individual types of collagen influence the ability of the ligament to withstand loading. Collagen I provides a great mechanical strength to connective tissues, whereas collagen III correlates with tissue elasticity. Therefore, a higher ratio of collagen I to III in the ligament is indicative of greater strength, whereas a lower ratio is characteristic of tissue laxity. Water accounts for 65-75% of the wet weight of ligament and much of this is associated with proteoglycans in the ECM. Together, water and proteoglycans have important lubricating and spacing roles that allow collagen fibres to glide over each other.
Extracellular Matrix

Connective tissues make up a large proportion of the total body mass. They are highly specialised and have a diversity of roles. They provide for mechanical support, movement, tissue fluid transport, cell migration, wound healing and control of metabolic processes in other tissues. Unlike the properties of epithelial, muscle or nerve tissues, which depend primarily on their cellular elements, the properties of connective tissues are determined primarily by the amount, type, and arrangement of an abundant ECM. It is not only of structural importance to hold cells and organs together; but also cells orient themselves through receptor-mediated interactions with specific components of the ECM. These interactions control cellular behaviour and morphology, and thus influence cell growth and differentiation.

Composition and Function: (Figure 1.2)

ECM consists of three major types of macromolecules; fibres, proteoglycans and glycoproteins, each of which is synthesised and maintained by cells specific to the tissue type. These cells include fibroblasts for fibrous connective tissue, Chondrocytes for cartilage and osteoblasts and osteocytes for bone. The two most important fibrous components of the ECM are collagen; the scaffold and framework of the ECM and elastin; the extensible element of the ECM, both insoluble macromolecular proteins. The striking feature of collagens is their ability to resist tensile loads with minimal elongation (less than 10%) under tension. A proportion of this elongation is not the result of true elongation of individual fibres, but of the straightening of fibres that are
Figure 1.2.

CT components
packed in various 3-dimensional arrays. In contrast, elastic fibres may increase their length by 150%, yet still return to their previous configuration. More than 20 genetically distinct collagens have been identified. Collagen I occurs throughout the body, except in cartilage. It is the principal collagen in the dermis, fasciae, tendons, and mature scar tissue. Collagen III dominates in the wall of blood vessels and hollow intestinal organs, and copolymerises with collagen I. The second major component of the ECM is the proteoglycans; a diverse group of soluble macromolecules that have both structural and metabolic roles. They form part of basement membranes and attach to cell surfaces where they function as receptors. Their mechanical functions include hydration of the matrix, stabilisation of collagen networks, and the ability to resist compressive forces, which is best exhibited in articular cartilage. The third group of matrix molecules, the glycoproteins, is ubiquitous in all connective tissues and, as with the proteoglycans, have both structural and metabolic roles. They provide linkage between matrix components and between cells and matrix.

Tenascins are large glycoproteins, which are involved in morphogenetic movements, tissue patterning, and tissue repair. They are “acute phase” proteins, which present transiently in the ECM but not expressed in normal adult tissue. They are generally found in adult tissues undergoing active remodelling, such as healing wounds and tumours. An immunohistochemical study revealed that tenasin was sparsely distributed in normal anterior cruciate ligaments, but strongly expressed in ruptured ligaments, suggesting that it may have a role to play during healing process after ligamentous injury. The first discovered member of the tenasin family, tenasin-
cytotactin (TN-C), is a multifunctional protein implicated in cell proliferation, migration, differentiation, and apoptosis. It was demonstrated that mechanical stress increased TN-C expression in fibroblasts.

**Cell adhesion to ECM:**

Cell adhesion to ECM is fundamental for maintaining normal tissue architecture and function. Changes in cell adhesion can occur as a result of modifications of the composition of the ECM, or as a result of disease associated changes in the expression and/or function of adhesion receptors. Such changes in cell adhesion may contribute to the loss of tissue architecture characteristic of POP. Collagens and cell surface proteoglycans have important roles in promoting cell adhesion. Specific types of collagen bind several distinct cell surface proteoglycans, including members of the syndecan family of heparan sulphate proteoglycans and the two major cell surface chondroitin sulphate proteoglycans (CSPGs): CD44/CSPG and NG2/CSPG. TN-C also modulates adhesion of cells to fibronectin, and it can be classified as an adhesion-modulating ECM protein.

The primary adhesion receptors for collagens and other constituents of ECM are members of the integrin group of the cell surface proteins. Integrins are a class of transmembrane glycoproteins that functionally link the cytoskeleton to the extracellular environment. They provide mechanical continuity between the outside and the inside of the cell. Integrins are implicated in both cell-matrix adhesion and cell-cell adhesion. Furthermore, integrin-mediated adhesion can activate intracellular signalling pathways,
which support cell survival and proliferation, and influence the expression of
differentiation-related genes. Each integrin consists of an α and a β subunit, and in
human there is 18 distinct α subunits and 8 β subunits which variously combine to form
24 functional receptors, each characterised by a distinct, although largely overlapping,
ligand-binding specificity.

Regulation of ECM:
Under normal physiological conditions, ECM is subject to constant remodelling
through a balance between synthesis and degradation. This balance is maintained
largely by stimulatory cytokines and growth factors, in addition to the degradative
matrix metalloproteinases (MMPs) enzymes and the tissue inhibitors of
metalloproteinases (TIMPs). The synthesis and secretion of MMPs and TIMPs
similarly modulated by an intricate network of signalling factors, cytokines, growth
factors and hormones. There are currently more than 20 characterised human MMPs,
whose function goes well beyond the ability to merely degrade structural ECM
proteins. They are major regulators of cell-cell and cell-ECM interactions in
physiological and pathological conditions that promote tissue turnover, including
development, hormone-dependent tissue remodelling, tissue repair, inflammation,
tumour invasion and metastasis. The alteration of the balance between synthesis and
degradation influences normal tissue architecture, impairs organ function and changes
the mechanical properties of the connective tissues. Moreover, alterations in the
metabolism of fibroblasts by local stimuli with growth factors or systemic hormonal
stimuli\textsuperscript{72} was found to have an influence on the quantity, type and stability of the collagen in the anterior cruciate ligament.

**ECM and pelvic floor disorders:**

Collagen is thought to contribute to the generation of urethral pressure profile. A correlation between urethral pressure measurements and skin collagen content was demonstrated by Versi \textit{et al.},\textsuperscript{73} who found better sphincteric function in association with greater collagen content of skin. It was suggested that the beneficial effect of oestrogen on urethral function might be mediated by collagen.\textsuperscript{73,74} Several investigators suggested that alterations in ECM are causally related to connective tissue disorders such as inguinal hernia\textsuperscript{43,44,75} and urinary stress incontinence.\textsuperscript{74} The increase of procollagen III mRNA, collagen III protein synthesis as well as MMP-1 and MMP-13 mRNAs and proteins might be the cause of recurrent inguinal hernias.\textsuperscript{43} Moreover, significantly lower amounts of collagen and higher amounts of elastic fibres were found in transversalis fascia from patients with direct inguinal hernia compared to those with indirect hernia. These changes might promote loss of resiliency of the transversalis fascia.\textsuperscript{76} Abdominal wall hernias could be similar to the herniation of the pelvic organs through the pelvic floor diaphragm.

The molecular mechanisms of human elastin gene regulation under various conditions remain largely unknown. Nevertheless, abnormal elastin production in inheritable or acquired diseases led to the loss of elastic recoil, and the resulting impaired integrity of elastic fibres played a major role in the clinical manifestations of a variety of
connective tissue disorders.\textsuperscript{77-79} It was also suspected to contribute to the development of POP.\textsuperscript{80} There are no data in the literature regarding the expression of tenascin in the pelvic floor of women with POP.

**Cytoskeleton**

The cytoplasm of eukaryotic cells is spatially organised by a network of protein filaments known as the cytoskeleton. This network contains three principal types of filaments; microtubules, actin filaments and intermediate filaments, which are connected to one another, and their functions are coordinated.\textsuperscript{81} Cytoskeletal proteins enable cells such as fibroblasts to generate large tractional forces that may ultimately influence tissue architecture.\textsuperscript{82}

Microtubules are stiff highly dynamic structures that alternately grow and shrink by the addition and loss of tubulin subunits, a protein that occurs in two forms, $\alpha$-tubulin and $\beta$-tubulin.\textsuperscript{81} Microtubules may occur anywhere in the cytoplasm and in any orientation, but the majority tend to radiate from the centrosome, which appears to be the microtubule organising centre of the cell. The number of microtubules in the cytoplasm varies according to the cell type and at different times in the same cell. They can be rapidly disassembled and reassembled in a new pattern to meet the cytoskeletal requirements of a change in cell shape.\textsuperscript{83}
Actin filaments are also dynamic structures, but they normally exist in bundles or networks rather than as single filaments. Actin is the most common contractile protein found within the cytoplasm of non-muscle. These filaments are most abundant in the ectoplasm (cortex) where they, jointly with a variety of actin-binding proteins, control the shape and surface movements, and contribute to the elasticity of the cells. In the endoplasm, they are less abundant but may aggregate in bundles that terminate at the cell membrane; the larger of these bundles are called stress fibres. The rapid conversion of actin between polymerised filamentous (F) and globular monomeric (G) forms is an important element in the dynamic changes of cell shape. The amount and distribution of F-actin in non-muscle cells vary with age, stage of differentiation and the agent with which the cells are stimulated.

**ECM-Actin Interaction**

The physical link between the ECM and the actin cytoskeleton occurs in focal adhesion, and is mediated by receptors of the integrin family through a large set of adaptor proteins. During cell migration, this physical link is dynamically modified, allowing the cell to sense and adapt to the microenvironment.

Actin cytoskeleton remodelling requires tight spatial and temporal regulation of actin filament assembly, which is accomplished by stimulating or inhibiting the activity of several actin-associated proteins. A fundamental step towards actin remodelling is the transmission of signals from the plasma membrane to the underlying actin. Integrin-
ligand binding initiates integrin-mediated intracellular signals in response to cell-matrix adhesion, which via a cascade of events, result in the remodelling of the actin cytoskeleton. These signal transduction pathways have profound effects not only on the structure and functional activity of the cytoskeleton, but also on cell survival and proliferation, and gene transcription. The cytoplasmic domain of integrins links with the actin filaments through a variety of bridging proteins, including profilin, gelsolin, vinculin, talin, α-actinin, tensin, zyxin, vinexin, nexillin and paxillin. The interaction with the actin leads to clustering of integrins and associated proteins into large multi-protein aggregates termed cell-matrix adhesions or focal adhesions at the cell surface, where actin filament ends are anchored to the plasma membrane and where bridging proteins are specifically localised. Because of this linkage across the plasma membrane, the cytoskeleton can anchor to the extracellular environment and control the tensional forces necessary for cell movement, cell-cell interactions, cell adhesion, migration, differentiation, as well as tissue development and genesis of diseases.

Several molecules are thought to transmit signals from plasma membranes to the actin cytoskeleton, such as the Rho family of small GTPases (Rho, Rac and Cdc42) and phosphatidylinositol 4,5 bisphosphate (PIP₂). Integrin-mediated cell adhesion regulates the activity of Rho GTPases. The small GTPases, in their active GTP-bound form, bind and activate a variety of effector proteins, which modulate the organisation of the actin cytoskeleton. Rho subfamily members promote stress fibres formation and focal adhesions, Rac mediates lamellipodia formation and Cdc42 induces filopodia formation and is required for the establishment of cell polarity during wound
healing. Rho induces stress fibre assembly primarily by the bundling of actin filaments, rather than actin polymerisation. Furthermore, the interplay between the family of small GTPases and PIP\textsubscript{2} metabolism controls signal transmission from the plasma membrane to the underlying actin. It was suggested that Rho subfamily\textsuperscript{95,101} and Rac\textsuperscript{102} might activate Phosphatidyl Inositol-4-Phosphate 5-Kinase. The PIP\textsubscript{2} formed predisposes the actin cytoskeleton to a “polymerisation mode” and assists its interaction with the plasma membrane by causing dissociation of G-actin from actin monomer binding proteins.\textsuperscript{103}

**Sex Steroid Receptors**

The diverse physiological activities of oestrogen are not restricted to the female reproductive system. It has been implicated in the function of most body systems, including central nervous system\textsuperscript{104} and bone.\textsuperscript{105} In addition to the positive effects, oestrogen plays a role in the development of uterine\textsuperscript{106} and breast\textsuperscript{107} cancers. The conflict between positive and negative activities of oestrogen has fuelled a search for selective oestrogen receptor modulators (SERMs) for use in hormone replacement therapy that possess the capability of harnessing the tissue selective beneficial effects of the steroids while lacking adverse activities in breast and uterus.\textsuperscript{108}

**Oestrogen Receptor (ER):**

Although oestrogens are among the most familiar hormones to both clinicians and patients, they are among the most mysterious through both their complex mechanisms
of action and multiplicity of roles. The discovery of at least two different forms of oestrogen receptors, together with the fact that some oestrogenic activities are non-genomic and possibly non-receptor mediated, may help to explain the broad spectrum of activity of the oestrogens and some of the apparently paradoxical effects of oestrogen-like drugs. It is also clear that the two oestrogen receptors genes are themselves regulated by oestrogen.\textsuperscript{109}

**Subtypes:** The classical oestrogen receptor, now called ER\(_\alpha\), is a protein of 595 amino acids and has a relative molecular mass of \(~66\) KDa.\textsuperscript{110} ER\(_\beta\) is a smaller protein consisting of 485 amino acids with a relative molecular mass of \(~54-55\) KDa.\textsuperscript{111} The two receptors are not isoforms of each other, but are rather distinct proteins encoded by separate genes located on different chromosomes; ER\(_\alpha\) is encoded on chromosome 6 while ER\(_\beta\) is encoded on chromosome 14.\textsuperscript{112}

**Structure:** Oestrogen receptors belong to the steroid hormone receptor family of nuclear hormone receptors that are a subset of the thyroid/steroid hormone receptor superfamily.\textsuperscript{113} The two ER subtypes have a similar structure. There are five distinct regions in oestrogen receptors termed A/B, C, D, E and F. The N-terminal A/B region contains a cell- and promoter- specific transcription activation function domain (AF-1) that can be activated in the absence of ligand.\textsuperscript{114} The C-region, that contains the DNA binding domain (DBD), has crucial amino acid sequences that create the two zinc fingers, which make direct contact with chromatin sequences. In addition, this region has phosphorylation sites\textsuperscript{115} and the dimerisation domain.\textsuperscript{113} The D region is called the
hinge domain because during receptor activation by ligand, a conformational change occurs in this region that is thought to simultaneously expose the nuclear localisation signal\textsuperscript{113} and the dimerisation domain.\textsuperscript{116} Finally, the C-terminal E/F region contains the ligand-binding domain (LBD). The E region binds the ligand and within the region are the ligand-dependent transcription activation function (AF-2) and the second dimerisation domain, which allows the formation of ER-ER dimmers.\textsuperscript{117} The role that F region plays in ER action is debatable, but it may help the stabilisation of the receptor-DNA complex to modulate the level of transcription induced by agonist-occupied ER.\textsuperscript{118} There is significant homology between these two receptor subtypes especially in the DBD (97\%) and in the LBD (59\%) but not elsewhere in the molecules.\textsuperscript{119} (Figure 1.3)

**Mechanism of action:** The classic oestrogen receptor pathway is thought to modulate the majority of oestrogenic effects. However, it is a relatively slow process and it can take hours or more to achieve its final downstream effect. It fails to explain some of the oestrogen induced actions, such as those affecting the excitability of nerve and smooth muscle cells which occur very rapidly, often within seconds of exposure.\textsuperscript{120} In the classical scenario, chaperone proteins (heat-shock proteins) hold the receptors in an inactive state, and when ligand binds, a conformational change occurs in the receptor and the heat-shock proteins are released and recycled. The receptors in their new conformational state translocate to the nucleus, form dimers that bind to the oestrogen response element,\textsuperscript{121} and regulate transcription and modulate the expression of target genes.\textsuperscript{122-124} Cells that contain both ER\textsubscript{α} and ER\textsubscript{β} proteins can create three possible
Figure 1.3

Structure of ER
trans-activating factor combinations, ER\(\alpha\) homodimers, ER\(\beta\) homodimers and ER\(\alpha\)–ER\(\beta\) heterodimers.\(^{125}\) The cell surface ER pathway was proposed to explain some of the more rapid effects of oestrogen on cells. The presence of ER on cell membranes would then act directly, or through a second messenger, to determine the flux of the sodium, potassium and calcium ions, which modulate the internal state of certain cells such as neurons.\(^{126}\) The other possibility that ER located on the cell membrane act through a genomic pathway, and this could explain certain actions in the central nervous system. This would require the indirect expression of a gene via a second messenger, such as a member of the cAMP response binding element superfamily.\(^{127}\)

**Progesterone receptor (PR):**

The PR is a member of the thyroid/steroid hormone receptor superfamily.\(^{128}\) PR expression is increased by oestrogen and decreased by progesterone.\(^{129}\) The physiological effects of progesterone are mediated by interaction with two receptor proteins, termed PR\(_A\) and PR\(_B\), that arise from a single gene and act as ligand-activated transcription factors to regulate the expression of reproductive target genes.\(^{130}\) Two distinct promoters have been identified that are independently regulated and give rise to the A and B isoforms.\(^{131}\)

**Structure:** The PR, as with all members of the nuclear receptor superfamily, is defined by a common structural motif that is organised into certain domains in terms of structure and function, namely; the amino terminal or A/B region,\(^{132}\) the DNA-binding domain (DBD) or region C,\(^{133}\) hinge domain or region D,\(^{134}\) and the ligand-binding
domain (LBD) or region E. PR_A and PR_B receptor isoforms differ only in that PR_B contains an additional fragment of amino acids located at the amino terminus of the receptor, and this region plays an essential role in specifying target genes that can be activated by the B but not the A protein.

Mechanism of action: Binding of progesterone to PR induces a significant conformational change on the heat shock proteins that allows stable receptor dimer formation, increased receptor phosphorylation, binding of receptor dimers to specific HRE located in the promoter regions of target genes, and interaction of the receptor complex with specific coactivator proteins and general transcriptional factors to form a productive transcription initiation complex on specific target gene promoters. PR_A and PR_B can dimerise and bind DNA as three species: A/A or A/B homodimers or A/B heterodimers depending on their ratio within the target cells under specific physiological conditions. Coactivators and corepressors also modulate progesterone-induced transcription through the ratio of coactivators to corepressors recruited to the transcription complex by promoter-bound receptors.

There is accumulating evidence that PR_A and PR_B are functionally different. PR_A seem to function as a transcriptional inhibitor of all steroid hormone receptors and a facilitator of ligand-dependent crosstalk among signalling pathways of sex steroid receptors within the cell. PR_B appears to be the transcriptional activator of progesterone-responsive genes. Thus the differential expression of both isoforms in
specific cell types may be critical for the appropriate cellular responses to progesterone.\textsuperscript{146-149}

**Androgen receptor (AR):**

The AR is a member of the thyroid/steroid hormone superfamily of nuclear receptors that function as ligand-activated transcription factors and influence cell function by regulating the transcription of androgen target genes.\textsuperscript{128} Unlike ERs, which are coded by two distinct genes,\textsuperscript{150} AR appears to be coded by a single copy gene localised on the X chromosome.\textsuperscript{151}

**Mechanism of action:** Most of the androgen functions are known to be mediated by specific binding to AR in target cells. Such ligand-receptor interaction results in conformational changes of the receptor protein, leading to a cascade of events that include dissociation of the receptor from heat shock proteins, dimerisation, receptor phosphorylation, translocation into the nucleus, binding to the androgen response elements on promoters of target genes, destabilisation of nucleosomal structure, and activation of the target gene transcription.\textsuperscript{152} On the other hand, there are some minor non-receptor-mediated pathways, such as its conversion to oestrogen by the enzyme aromatase in certain target cells\textsuperscript{153} and androgen interaction with circulating sex hormone-binding globulins.\textsuperscript{154}

**AR in the females' ligaments:** No human or animal studies so far investigated the expression of AR in females' pelvic ligaments. AR, however, were identified in the
human anterior cruciate ligament of young men but not in women of any age group. The administration of testosterone in orchiectomised male rats significantly increased collagen content and collagen fibril diameter of the hip joint capsule. However, they were increased in female rate by ovariectomy and decreased by the administration of oestrogen alone, or oestrogen combined with progesterone.

The role of oestrogen in pelvic floor function:

Human studies found that oestrogen receptors are expressed in the squamous epithelial cells of the female urethra and trigone of the bladder, fallopian tubes, uterosacral ligaments, endometrium, vagina, levator ani muscle, and the vulva. The presence of oestrogen receptors in the pelvic floor and the urogenital ligaments suggest that these tissues are targets for oestrogen action. Oestrogen treatment stimulated proliferation of the vaginal and urethral epithelia, increased the blood supply and relieved the symptoms associated with atrophy. Both oral and topical oestrogen preparations were effective, but administration of oestrogen vaginally was found to be more appropriate than oral administration for treatment of urogenital symptoms. It was suggested that oestrogen improves urethral sphincter function by increasing the volume of submucosal venous plexuses in the urethra and by effects on collagen metabolism. Nonetheless, studies on oestrogen treatment of urinary incontinence in postmenopausal women reported variable results. Faber and Heidenreich found that oral oestrogen therapy significantly increased urethral pressure profile, but Walter et al. found no change, and stress incontinence was unaffected. There was, however, some improvement in symptoms of frequency, urgency and urge incontinence.
Levormeloxifene and POP:

Levormeloxifene, the L-enantiomer of racemic centrochroman (ormeloxifene), is one of the selective oestrogen receptor modulators (SERMs) that make up a class of pharmacologic agents with both oestrogenic and anti-oestrogenic properties depending on the target tissue and hormonal milieu.\textsuperscript{108,168} In a clinical trial of healthy postmenopausal women, levormeloxifene exhibited beneficial effects on bone mineral density and lipid profile.\textsuperscript{169} Within two years, its clinical development was discontinued because of the concerns over endometrial stimulation and increased POP with urinary incontinence.\textsuperscript{170} A higher proportion of surgery for POP in treated versus untreated women was noted.\textsuperscript{168} Similar, unpublished, side effects were observed with idoxifene, which belongs to triphenylethlenes; another class of SERMs.\textsuperscript{168} The possible role of these SERMs in the pathogenesis of POP was not investigated before.

Mechanical Stretch

Fibroblasts are subjected to changes of the mechanical force balance \textit{in vivo} during physiological as well as pathological conditions, such as wound healing and development of hypertrophic scars. Many \textit{in vitro} and animals studies have demonstrated the capacity of stretch to modulate cell behaviour through several different signalling pathways. A variety of cell types was used, including vascular endothelial cells,\textsuperscript{171} fibroblasts,\textsuperscript{172-177} and lung epithelial cells.\textsuperscript{178} Most of the studies performed \textit{in vitro} utilised the culture of cell monolayers on matrix-bonded elastomeric
membrane substrates that were deformed to impose well-characterised stretch, offering the advantage of a controlled environment.

Application of stretch in vitro modified fibroblast proliferation, influenced the differentiation state of fibroblasts, which developed prominent α-smooth muscle actin-containing stress fibres resembling myofibroblasts, changed fibroblast morphology, changed collagen I and III mRNA expression in knee ligament fibroblasts, induced a significant increase in collagen α1 mRNA and fibronectin protein synthesis by vascular smooth muscle cells, which was significantly attenuated by losartan (AT1-receptor antagonist), increased production of TIMP-1 by scleral fibroblasts, induced nitric oxide production in human patellar tendon fibroblasts, enhanced secretion of interleukin 6 in human tendon fibroblasts, and induced adaptations in gene transcriptional profile. Animal studies showed that stretch could also increase the inflammatory reaction and damage repair tissue, leading to failure of the healing process. Nevertheless, the effect of stretch on the transcriptional profile and morphology of the cytoskeleton of fibroblasts derived from cardinal ligaments was not studied before.

Microarrays Technology

Background:
Various methods are available for detecting and quantifying gene expression levels, including Northern blots, S1 nuclease protection, differential display,
sequencing of cDNA libraries\textsuperscript{187} and serial analysis of gene expression.\textsuperscript{188} Augmenting this coterie are two array-based technologies; cDNA and oligonucleotide arrays. Microarray technology allows simultaneous measurement of the expression level of thousands of genes in a single hybridisation assay.\textsuperscript{189} They allow the study of expression levels in parallel, thus providing static information about gene expression (i.e. in which tissue(s) the gene is expressed) and dynamic information (i.e. how the expression pattern of one gene relates to those of others).\textsuperscript{190} Microarray based studies are uncovering broad patterns of genetic activity, providing new understanding of gene functions, and generating insight into transcriptional processes and biological mechanisms.\textsuperscript{191} The range of microarray applications is expanding. Emerging from their roots in gene screening and target identification, microarrays are now being applied to disease characterisation, developmental biology, pathway mapping, mechanism of action studies and toxicology. Scientists are conducting profiling studies that may lead to the use of microarrays in molecular diagnosis of disease, and in predicting drug efficacy and toxicity in different individuals.\textsuperscript{192}

**Technology overview:**

Several methods have been described for producing microarrays. In cDNA microarrays, which are also called spotted microarrays, microscope slides containing hundreds to thousands of immobilised DNA samples are hybridised in a manner very similar to the Northern and Southern blot.\textsuperscript{193,194} Templates for genes of interest are amplified by polymerase chain reaction, and printed on coated glass microscope slides using a computer-controlled, high-speed robot. Each array consists of a reproducible pattern of
thousands of different DNAs. The relative abundance of the spotted DNA sequences in two mRNA samples can be addressed by monitoring the differential hybridisation of the two samples to the sequences on the array. Two mRNA samples or targets are reverse transcribed into coda and labelled by incorporating fluorescently tagged nucleotides during oligo-primed reverse transcription of mRNA. The frequently paired fluorescent labels, Cye5-dUTP and Cye3-dUTP, allow highly discriminating optical filtration not only because of their high incorporation efficiencies with reverse transcriptase, good photostability and yield, but also because they are widely separated in their excitation and emission spectra. After competitive hybridisation with arrayed DNA sequences or probes, the slides are imaged using a scanner, and fluorescence measurements are made separately for each dye at each spot of the array. Laser excitation of the incorporated targets yields emissions with characteristic spectra, which are measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo-coloured and merged. Information about the clones, including gene name, clone identifier, intensity values, intensity ratios, normalisation constant and confidence intervals is attached to each target. The ratio of the fluorescence intensities for each spot is indicative of the relative abundance of the corresponding DNA probe in the two nucleic acid target samples. Data from a single hybridisation experiment is viewed as a normalised ratio [Treatment (T)/Control (C)] in which significant deviations from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression relative to the reference sample. In addition, data from multiple experiments are examined using any number of data mining tools. It is a key issue to decide whether to use direct or
indirect comparisons i.e. to compare between gene expression in two cell-population samples, T and C, within or between slides. In a direct comparison, the differential expression of the genes in samples T and C is measured directly on the same slide (in a single experiment). In an indirect comparison, expression levels of samples T and C are measured separately on two different slides in comparison with a common reference RNA (R). The purity of RNA is a critical factor in hybridisation performance, particularly when using fluorescence, as cellular protein, lipid and carbohydrate can mediate significant non-specific binding of fluorescently labelled cDNAs to slide surfaces. The clear limitation to the application of cDNA technology is the large amount of RNA required per hybridisation. For adequate fluorescence, the total RNA required per target per array is 50-200 μg (2-5 μg only are required when using Affymetrix).

High-density oligonucleotide microarray experiments, also called oligo chips or GeneChip® oligonucleotide arrays (Affymetrix, Inc, Santa Clara, CA), provide direct information about the expression levels in a mRNA sample of the 200,000-500,000 probed gene fragments. A given gene is represented by 15-20 different 25-mer oligonucleotides that serve as unique, sequence-specific detectors. An additional control element on these arrays is the use of mismatch (MM) control oligonucleotides that are identical to their perfect match (PM) partners except for a single base difference in a central position. The presence of the MM oligonucleotide allows cross-hybridisation and local background to be estimated and subtracted from the PM signal. In the GeneChip expression assay, eukaryotic mRNA is converted to
biotinylated cRNA from oligo-dT-primed cDNA.\textsuperscript{197} Each sample is hybridised to a separate array. Transcript levels are calculated by reference to cRNA spikes of known concentration added to the hybridisation mixture. Differences in mRNA levels between samples are determined by comparison of any two hybridisation patterns produced on separate arrays of the same array type.\textsuperscript{196}

There are important differences in performance and information generated by the two types of arrays. First, The Affymetrix data measure gene expression levels for each sample separately, whereas cDNA experiments are based on competitive hybridisation between two samples. This leads to relative expression values (expression ratios) and constrains the types of design that can be considered. The Affymetrix, on the other hand, allows flexibility in sample comparisons and provides an estimate of the levels of gene transcripts in individual samples. Second, on Affymetrix the oligomer probes are designed to uniquely represent the cognate gene thus minimising cross- hybridisation between similar sequences. The potential for cross- hybridisation between genes with significant levels of sequence similarity is high using cDNA microarrays. Finally, the Affymetrix requires gene sequence information for specifying the de novo synthesis of the oligomers on the array, whereas cDNA arrays are produced from both known and unknown cDNA and PCR fragments.\textsuperscript{191}

**Design issues:**

The objective of experiment design is to make the analysis of the data and the interpretation of the results as simple and as powerful as possible, given the purpose of
the experiment and the constraints of the experimental materials. Good design is crucial to ensure best use of the available resources and to avoid obvious biases. A badly designed experiment might leave an experimenter unable to answer a question of interest with the data that was collected, or may leave a potential bias in the data that might compromise the interpretation of the results.\textsuperscript{198} Table 1.2 contains a list of general issues that need to be addressed when designing a microarray experiment.

\textbf{Variability and replication:}

Replication is an essential feature of comparative microarray experiments. The cDNA microarray system is rather variable at the individual gene level. Expression of a gene might vary four-fold in one hybridisation, but only 1.3 fold in a second independent hybridisation, and two-fold in a third. Therefore, replication is essential to minimise the chance of getting false positive results and to allow averaging. Averages are less variable than their component terms. In addition, replicates reduce variability in summary statistics (defined as a numerical summary of some aspect of an experiment, typically an estimate of a parameter).\textsuperscript{189} It is difficult to say how many replicates should be done, although some investigators indicate that two\textsuperscript{199} or three are sufficient.\textsuperscript{200} There are several forms of replication. Given that replicate hybridisations are almost invariably carried out by the same person, using the same equipment and protocols, and frequently at about the same time, it is inevitable that replicate data will share many features. \textbf{Technical replicates} between slides refers to replication in which the target mRNA is from the same pool i.e. from the same extraction. Technical replicates generally involve a smaller degree of variation in measurements than the biological
Table 1.2: General issues that affect the design of array experiments

**Scientific constraints:**

- Aim of the experiment: whether to identify differentially expressed genes, search for specific gene expression patterns, or to identify tumour subclasses.
- Specific questions to be answered and how they are prioritised.
- How will the experiments answer the posed questions?

**Logistic constraints:**

- Types of mRNA samples: reference, control, treatment, and so on.
- Amount of RNA expected to be available.
- Number of the slides available for the experiment.

**Other factors:**

- The experimental process before hybridisation: sample isolation, mRNA extraction, amplification and labelling, which will affect the number of replicates required.
- Controls planned: positive, negative, ratio and so on.
- Verification method: northern blot, reverse transcriptase (RT)-PCR, or *in situ* hybridization.
- Cost considerations.
replicates. Consequently, they do not provide the independence of data that gives the fullest benefits of averaging and this reduces their value for broader statistical inference. **Biological replicates** usually refer to hybridisation that involves either mRNA from the same subject but from different extractions i.e. from different samples of cells or mRNA from different subjects. This form of replicates typically involves a much greater degree of variation in measurements. The type of replication to be used in a given experiment affects the precision and the generalisability of the results. Biological replicates are used to obtain averages of independent data and to validate generalisation of conclusions. However, technical replicates are used to assist in reducing the variability. For example, if a conclusion that is applicable to all mice of a certain inbred strain is sought, experiments that involve many randomly selected mice must be carried out. Extrapolating to all mice of that strain from results on a single mouse, even when several mRNA extractions are used, is of limited value.\(^{189}\)

**Data analysis strategies:**

Microarray experiments generate large and complex multivariate data sets, and some of the greatest challenges lie not in generating these data but in the development of computing and statistics tools to analyse the large amounts of data. The data format typically consists of a list of genes and corresponding values that represent relative RNA transcript levels. A small-scale experiment consisting of only five samples with two replicates each will produce approximately 100 000 data points.\(^{201}\) There are three basic steps required for efficient data analysis; data normalisation, data filtering and pattern identification. To compare expression values directly, it is usually necessary to
apply some sort of normalisation strategy to the data, either between paired samples or across a set of experiments, because it is important to distinguish real biological change from random noise or non-specific experimental variation. Therefore, the value of doing replicate experiments cannot be overstated. Following this, data reduction can be done by filtering out uninformative genes; for example, genes that are expressed below a user-defined threshold or genes that did not vary their expression level during the course of the experiment. The next step is to find patterns and groups in the data that can be used to assign biological meaning to the expression profiles.199

The methods used for data mining and interpretation are varied, ranging from straightforward lists of increased and decreased genes based on user-defined thresholds to the implementation of sophisticated clustering and visualisation programmes, such as hierarchical clustering202 and self-organising maps also called k-means clustering.203 The data mining strategy used depends on the experiment design and is broadly divided into two categories: differential gene expression and coordinated gene expression. The differential gene expression approach generally consists of paired comparisons between normal/abnormal data such as from healthy and pathological specimens. Coordinated gene expression analysis involves the assessment of the expression levels of a large number of genes over a period of time or through a series of experimental conditions.199
Chapter Two

Aims of the research programme
The high incidence of POP observed in postmenopausal women and in those using SERMs, suggested an aetiological role for the hypoestrogenic state. Since oestrogen deficiency is considered to be the key event that affects ECM, and given the well-observed oestrogen-related molecular and structural changes in different ligaments in human and animal studies, it was predicted that POP would be associated with alterations in the ECM composition of the cardinal ligaments. The previous findings of reduced CI/CIII ratio with increased laxity of the knee ligaments, down-regulated elastin synthesis by fibroblasts from prolapsed cardinal ligaments, and raised tenasin expression in the ruptured knee ligaments, led to the hypothesis that prolapsed cardinal ligaments would have higher expression of CIII and tenasin, and lower expression of CI and elastin, and HRT would reverse these changes in postmenopausal women. To test this hypothesis, a series of immunohistochemical studies were conducted to document for the first time the differential expression of four ECM proteins; collagen I, collagen III, elastin and tenasin in human cardinal ligaments of prolapsed uteri compared to non-prolapsed controls in premenopausal and postmenopausal women, and also in postmenopausal women treated with sequential combined HRT.

The interaction between ECM proteins and oestrogen, and the regulation of oestrogen action by ECM proteins was well observed. Moreover, in a study of varicose veins, a higher expression of the ERα and PR was found in the mechanically failed varicose segment compared to the adjacent healthy segment, suggesting that steroid receptors could be invoked in the sustenance of structural integrity and functional competence of
the ECM. These observations led to the hypothesis that the expression of the oestrogen receptors would be elevated in the prolapsed cardinal ligaments as a compensatory response to the oestrogen deficiency induced alterations in the ECM. Ovarian steroids were known to interact with and influence the concentration of each other, and a high concentration of either the cross-reactive ligand or the unliganded receptor evoked a receptor-specific biological response. Therefore; it was anticipated that, at least in the postmenopausal state, the prolapsed cardinal ligaments would be in a “starvation state” and expressing higher level of all gonadal steroids receptors. This led to the hypothesis that PR and AR expression would also be increased in the prolapsed cardinal ligaments. Furthermore, it was hypothesised that HRT would have ameliorating effects. To test these hypotheses, another series of immunohistochemical studies were conducted to investigate the differential expression of gonadal steroids receptors; ERα, ERβ, PR and AR in human cardinal ligaments of prolapsed uteri compared to non-prolapsed controls in premenopausal and postmenopausal women, and also in postmenopausal women treated with sequential combined HRT. Further, since one of the possibilities for oestrogen’s mode of action could be ascribed to its proliferating effect on fibroblasts, the density of Ki-67 positive cells, as a surrogate marker of proliferative activity, was analysed to determine whether the detected changes were associated with an increased proliferative activity of the fibroblasts or were a prolapse-related phenomenon.

The immunohistochemical findings of this research project supported by the previous findings of elevated expression of ERα and PR, and tenascin by mechanical
stretch led to the hypothesis that the expression of a number of genes representing the altered ECM proteins and gonadal steroids receptors in the cardinal ligaments would be modified. It was also reasonable to assume that the expression of other genes coding for proteins involved in maintaining the cellular and extracellular integrity could also be changed. The marked inter-subject variability and the impossibility of obtaining large enough cardinal ligament specimens to collect adequate amount of RNA per sample were the leading factors to rely on the fibroblast culture technique rather than extracting RNA from ligament specimens directly. Given the fact that the prolapse is a state of tissue collapse that results from a process of excessive stretch of the cardinal ligaments, it was assumed that the effect of mechanical stretch on primary cultures of fibroblasts derived from the cardinal ligaments in vitro would be similar to the effect of chronic stretch associated with prolapse in vivo. To test the hypothesis, a study was designed to examine the changes in the transcriptional profile of fibroblasts derived from cardinal ligament and grown in primary cultures, in response to exposure to mechanical stretch, using cDNA microarray technology.

The cDNA microarray study revealed alterations in a number of mechano-responsive genes involved in the regulation of actin cytoskeleton remodelling and cell-matrix interactions. Nonetheless, it fell short of identifying alterations in gene expression commensurate with the findings obtained from the immunohistochemical studies. In addition, it was previously reported that in vitro mechanical stretch changed fibroblast morphology, which appeared elongated, bipolar, and oriented along the lines of tension, while they appeared stellate with short processes in relaxed collagen lattices,\textsuperscript{176}
suggesting a detrimental effect for mechanical stretch on the cytoskeleton morphology. This was a major turning point in this research project, and led to the hypothesis that the mechanical strain of cardinal ligaments, e.g. in cases of increased intra-abdominal pressure, could cause POP by destroying the cytoskeleton of the fibroblasts. It was also plausible to assume that SERMs induced POP in healthy asymptomatic women by the same mechanism, and that oestrogen would prevent and/or reverse this effect and maintain the integrity of the fibroblasts. To test these hypotheses, the work was extended to study the effect of mechanical stretch, 17β-oestradiol and levormeloxifene on the morphology of the cytoskeleton (α-tubulin and F-actin) of those fibroblasts grown in primary cultures. Moreover, Stanley and colleagues documented a significant attenuation of the stretch-induced rise in collagen α1 mRNA and fibronectin protein synthesis by vascular smooth muscle cells, which were treated with losartan (AT1-receptor antagonist). Therefore, another experiment was designed to seek for possible protective effect of losartan on the actin cytoskeleton of stretched fibroblasts in primary culture.
Chapter Three

Materials and Methods
The investigation protocol was approved by the Leicestershire Ethics Committee and every patient signed an informed consent form preoperatively, allowing the use of tissues removed at surgery for research purposes.

3.1. Immunohistochemical studies

Demographic data:
Thirty-three cardinal ligament samples were obtained from women with POP; 8 from premenopausal and 25 from postmenopausal women, 10 on HRT and 15 not taking HRT. The duration of the prolapse was not known precisely due to the insidious nature of the problem. Twenty-five control samples were taken from women with no prolapse, as assessed pre-operatively; 15 from premenopausal and 10 from postmenopausal women not on HRT. All specimens were taken from Caucasian women. All women with prolapse have had vaginal hysterectomy (except two cases in the premenopausal group who have had abdominal hysterectomy and sacral colpopexy), while those with no prolapse have had abdominal hysterectomy. All the cases of prolapse included in this study were those with advanced POP stage III or IV, where the uterine isthmus reached the introitus. None of these women used a supportive vaginal pessary or ring prior to surgery. The menopause was defined as one year of amenorrhoea in women over the age of 45. All postmenopausal women in this study met this criterion. All HRT users were taking sequential combined HRT (oestradiol valerate 2 mg daily and cyclic norethisterone 1 mg per day for 12 days of each 28-day treatment cycle) for at least 6 months pre-operatively. Similar groups were compared together to evaluate the effect
of exogenous gonadal steroids after the menopause, the effect of the menopause per se and the effect of prolapse in a mixed model; (i) postmenopausal women with prolapse on HRT versus no HRT, (ii) postmenopausal women not on HRT with prolapse versus no prolapse, and (iii) premenopausal women with prolapse versus no prolapse. It would have been interesting to obtain specimens from postmenopausal women who were receiving HRT and not suffering from prolapse, but there would be no pretext as to why we can access their tissues other than scientific curiosity. This group of women are usually free from gynaecological problems and at low risk to have hysterectomy. The demographic data of subjects are summarised in table 3.1.

**Specimens:**

Five millimetre thick slices of the medial ends of the cardinal ligament were obtained from the part of the cervix above the portio vaginalis (Figure 3.1). Samples were immediately fixed in 10% formal saline for 24 h, embedded in paraffin wax, and 5 μm sections were cut using a Leica RM2035 microtome, mounted onto silane-coated slides (3-Aminopropyl-Triethoxy-Silane, Sigma, Dorset, UK) and allowed to dry at 37°C for 48 h. This technique was previously optimised in our laboratory.\(^\text{217,218}\)

**Haematoxylin and Eosin (H&E):**

All sections were stained with H&E for histological assessment of the ligamentous tissue. Tissue sections were dewaxed in xylene, rehydrated in 99%, 90%, 70% ethyl alcohol (Industrial Methylated Spirit, IMS; Genta Medical, York, UK), washed in distilled water, stained with Harris haematoxylin (Sigma, Dorset, UK), and washed in
Table 3.1: The demographic attributes of women included in the immunohistochemistry studies

<table>
<thead>
<tr>
<th></th>
<th>Prolapse</th>
<th></th>
<th>No Prolapse</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PrM</td>
<td>PM</td>
<td>PM/HRT</td>
<td>PrM</td>
</tr>
<tr>
<td>Number of cases</td>
<td>8</td>
<td>15</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Age (mean &amp; 95% CI)</td>
<td>45.8 (40.6,50.9)</td>
<td>66.2 (61.5,70.9)</td>
<td>60.0 (55.4,64.6)</td>
<td>42.3 (38.0,46.7)</td>
</tr>
<tr>
<td>Parity (mean &amp; 95% CI)</td>
<td>2.4 (1.9,2.8)</td>
<td>2.5 (1.7,3.3)</td>
<td>2.7 (2.0,3.3)</td>
<td>2.3 (1.4,3.1)</td>
</tr>
</tbody>
</table>

CI = confidence interval
PrM = premenopausal
PM = postmenopausal
PM/HRT = postmenopausal on HRT
Figure 3.1.

Specimen Collection
running tap water. Sections were then stained in Eosin (TAAB laboratories, Berkshire, UK), washed in running water before dehydration in alcohol in reverse order to the rehydration steps. All the previous steps were done for 5 min each. The slides were mounted using DPX mounting medium (contains distrene 80, dibutyl phthalate and xylene). Histological examination revealed no discernable difference between prolapsed ligaments and controls (Figure 3.2).

**Antibodies:**

The primary and secondary antibodies used in IHC are listed in Table 3.2 and 3.3, respectively.

**IHC protocols:**

The immunostaining for steroid receptors and Ki-67 was carried out using standard protocols previously optimised in our laboratory with some modifications.\textsuperscript{218-220} The staining technique for the ECM proteins was optimised after reviewing the data sheets and the websites of the manufacturers. To determine the optimal concentration of each primary antibody, serial dilutions were used in pilot studies on test specimens. The highest dilutions, which showed differences in staining patterns, on microscopic examination, between the prolapse and no prolapse specimens were used. The anti-tenascin antibody, used in this study, recognises all isoforms of human tenascin.
Figure 3.2.

H&E
### Table 3.2: The primary antibodies used in immunohistochemistry and α-tubulin staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Clone No.</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Monoclonal mouse anti-ovine</td>
<td>6F11</td>
<td>1:50 in blocking solution</td>
<td>Novacastra, Newcastle upon Tyne, UK</td>
</tr>
<tr>
<td>ERβ</td>
<td>Polyclonal rabbit anti-rat</td>
<td>(Catalogue no. 06-629)</td>
<td>1:50 in blocking solution</td>
<td>Upstate Biotechnology, Lake Placid, NY, USA</td>
</tr>
<tr>
<td>AR</td>
<td>Monoclonal mouse</td>
<td>AR-27</td>
<td>1:25 in blocking solution</td>
<td>Novacastra, Newcastle upon Tyne, UK</td>
</tr>
<tr>
<td>PR</td>
<td>Monoclonal mouse</td>
<td>1A6</td>
<td>1:40 in blocking solution</td>
<td>Novacastra, Newcastle upon Tyne, UK</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Monoclonal mouse</td>
<td>B56</td>
<td>1:150 in blocking solution</td>
<td>Pharmingen, Palo Alto, USA</td>
</tr>
<tr>
<td>Collagen-I</td>
<td>Monoclonal mouse</td>
<td>COL-1</td>
<td>1:6000 in blocking solution</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Collagen-III</td>
<td>Monoclonal mouse</td>
<td>HWD1</td>
<td>1:50 000 in blocking solution</td>
<td>InnoGenex, California, USA</td>
</tr>
<tr>
<td>Elastin</td>
<td>Monoclonal mouse</td>
<td>BA-4</td>
<td>1:1600 in blocking solution</td>
<td>Novacastra, Newcastle upon Tyne, UK</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Monoclonal mouse</td>
<td>BC-24</td>
<td>1:100 000 in blocking solution</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Fluorescent monoclonal mouse</td>
<td>B-5-1-2</td>
<td>1:2000 in 3% BSA/PBS*</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>

*BSA/PBS = bovine serum albumin/phosphate buffered saline  ERα = oestrogen receptor α  ERβ = oestrogen receptor β  AR = androgen receptor  PR = progesterone receptor
Table 3.3: The secondary antibodies used in immunohistochemistry and α-tubulin staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>ERβ</td>
<td>Biotinylated swine anti-rabbit</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>AR</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>PR</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Biotinylated goat anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Collagen-I</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Collagen-III</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Elastin</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Biotinylated rabbit anti-mouse</td>
<td>1:400 in blocking solution</td>
<td>Dako A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Fluorescent labelled rabbit anti-mouse</td>
<td>1:200 in 3% BSA/PBS*</td>
<td>Molecular Probes, Leiden, The Netherlands</td>
</tr>
</tbody>
</table>

*BSA/PBS = bovine serum albumin/phosphate buffered saline  
ERα = oestrogen receptor α  
ERβ = oestrogen receptor β  
AR = androgen receptor  
PR = progesterone receptor
3.1.1. IHC for ERα:

1- Sections were de-waxed in xylene (Genta Medical, York, UK) in three steps for 3 min each.

2- Sections were re-hydrated in 99%, 99% and 95% industrial methylated spirit (IMS, Genta Medical, York, UK) and distilled water for 3 min each.

3- Microwave antigen retrieval was performed in 10 mmol citrate buffer, pH 6.0, at 700 W for 15 min, followed by cooling for 20 min, and then washing in tap water.

4- Endogenous peroxidase activity was quenched by incubating the sections in freshly prepared, cold hydrogen peroxide 6% v/v in distilled water for 10 min, followed by two washes in de-ionised water and phosphate buffered saline (PBS)-Tween 20 (0.05% v/v) for 5 min each.

5- Sections were blocked with normal rabbit serum in 3% bovine serum albumin (BSA) in PBS (NRS, diluted 1:10, Dako, Glostrup, Denmark), 100μl per section, at RT, in a humidity chamber, for 30 min, to minimise non-specific reactivity.

6- Further blocking was performed with Avidin-biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA). 100μl per section of Avidin solution (prepared by adding 4 drops of Avidin to 1 ml of blocking solution) was added for 15 min at RT, followed by a 5 min wash in PBS-Tween 20, then 100μl per section of Biotin solution (prepared by adding 4 drops of Biotin to 1 ml of blocking solution) was added for 15 min at RT, followed by another 5 min wash in PBS-Tween 20.

7- Slides were incubated overnight at 4°C with the primary antibody (100μl per section) in a humidified chamber.
8- After washing in PBS-Tween 20 for 20 min, sections were incubated for 30 min at RT with the secondary antibody (100μl per section) in a humified chamber.

9- After a further 20 min wash in PBS-Tween 20, sections were incubated at RT with Vectastain ABC peroxidase (Elite; Vector Laboratories Ltd., Peterborough, UK) for 30 min. Vectastain ABC peroxidase solution was mixed, at least 30 min before use, by adding 2 drops of solution A and 2 drops of solution B to 5 ml of PBS. Solution was added at 100μl per section.

10- Slides were washed in PBS-Tween 20 for another 20 min, and bound antibodies were visualised by incubation for 5 min with 0.05% diaminobenzidine (DAB) in 0.05 mol/l Tris-HCl, pH 7.4, and 0.01% hydrogen peroxide according to the manufacturer's instructions (Vector Laboratories Inc., Burlingame, CA, USA). Sections were then washed in running tap water for 5 min.

11- Slides were submerged in CuSO₄/NaCl (16mmol/l/123mmol/l) solution for 5 min to enhance DAB stain, then washed in distilled water for 5 min.

12- Counter-staining with haematoxylin, Gill’s formula (Vector Laboratories Inc., Burlingame, CA, USA) for 1 min was performed to improve identification of cellular elements, followed by rewashing in tap water. Slides were submerged in acetic acid 2% for 3 min, then in tap water for a few seconds.

13- Sections were dehydrated by submersion in 95%, 99% and 99% alcohol for 3 min each and cleared in xylene overnight.

14- Slides were permanently mounted using XAM® neutral mounting medium (Waters and Rogers Van International Limited, Poole, Dorset, UK) and covered with the appropriate cover slip.
3.1.2. **IHC for ERβ**: was similar as above, but blocking was with normal swine serum (NSS, diluted 1:10, Dako, Glostrup, Denmark).

3.1.3. **IHC for AR**: was as above with the following differences:

- In step 3: Microwave antigen retrieval was performed in 1 mmol EDTA solution, pH 8.0, at 700 Watts for 12 minutes.
- In step 4: Endogenous peroxidase activity was quenched by incubating the sections in freshly prepared, cold hydrogen peroxide 1.5% in methanol for 10 min.
- Tris-buffered saline (TBS) rather than PBS was used for preparation of blocking solution and washing in all steps.

3.1.4. **IHC for PR**: was similar as above, but Avidin-Biotin blocking was omitted.

3.1.5. **IHC for Ki-67**: was similar as above, but blocking was with normal goat serum (NGS, diluted 1:20, Dako, Glostrup, Denmark).

3.1.6-9. **IHC for Collagen I & III, Elastin and Tenascin**: was as above with the following differences:

- Antigen retrieval was done using trypsin digestion:
  - 300 ml of PBS and 300 ml of distilled water were pre-heated to 37°C.
  - Slides were preheated to 37°C in the distilled water for at least 5 min.
  - 0.3g Trypsin 1:250 (Difco Laboratories, Detroit, Michigan, USA) and 0.3g Calcium Chloride (Sigma-Aldrich, Dorset, UK) were dissolved in
the pre-heated PBS, and pH was adjusted to 7.8. Slides were submerged in this solution for 30 min.

- Slides were then rinsed in running tap water for 5 min.

-Avidin-Biotin blocking was omitted.

Quality control in IHC:

Specificity of immunostaining was confirmed using non–specific mouse immunoglobulin (mouse IgG, 1:2000, Vector Laboratories Inc., Burlingame, CA, USA) alongside all experiments except for ERβ where rabbit IgG (1:1000, Vector Laboratories Inc., Burlingame, CA, USA) was used. There was no background reaction in any slide. Negative control sections were produced by omission of the primary antibody and did not show any staining, while the positive control slides were stained with the primary antibody.

Evaluation of the tissue sections:

The methods used are detailed in the relevant chapters.

Numbers of fields assessed:

The number of fields per slide required to be examined when detecting each antigen was assessed, in order to account for the variability within specimens. The aim was to detect a statistically significant difference of $\alpha=0.05$ (5% significance) and $\beta=0.8$ (80% power). The following equation was used:

$$\frac{1}{N_s} \times (V_s + V_f/N_f)$$
Ns = number of slides \hspace{1cm} V_s = variance between slides

V_f = variance between fields \hspace{1cm} N_f = number of fields

**Assessment of reproducibility of the histomorphometric measurements:**

The reproducibility of the histomorphometric measurements and count was evaluated using Bland and Altman analysis. The Bland-Altman plot, also known as “difference plot” or “method-comparison plot”, is used to assess agreement between two methods of measurement. The graph is constructed so the difference between the two methods of measurements (Y axis) is plotted against the average obtained with each of the two techniques (X axis). Further, Bland-Altman analysis is used to check the repeatability of a single measurement method or to compare measurements by two observers.\(^{221}\)

To evaluate the reproducibility of the count of positive and negative nuclei in the steroid receptors study, 12 slides were chosen at random to be examined by a second observer. Each observer produced a mean value of measurements for each slide, and a Bland-Altman plot for the inter-observer variability was generated (Figure 3.3). The difference between the two observers was 1.2%, which was statistically non-significant. Similarly, the reproducibility of the measurements of the stained areas of the ECM was evaluated using the same statistical method (Figure 3.4). The difference between the two observers was 15%, but the measurements were consistent throughout. Bland-Altman plots for the intra-observer (for the main observer; ATEE) variability were also generated. Six slides were chosen at random to compare the count of positive and negative nuclei in the steroid receptors study in two different settings. The results
Figure 3.3

Bland-Altman (SR)-inter-observer
Figure 3.4

Bland-Altman (ECM)-inter-observer
are shown in Figure 3.5. There was no evidence of bias (p=0.85). There was no percentage error, and the 95% limits for proportion of positive were ±0.065 i.e. in the 95% of the times, the difference in counting positive nuclei between the two observations was ±0.065. This was statistically non-significant. Similarly, Six slides were chosen at random to compare the measurements of the stained areas of the ECM in two different settings. There was no evidence of bias (p = 0.27). The 95% agreement between the two observations was within ±15%. However, the difference was dependent on the size of the measurement i.e. percentage error, which is common and not of much concern (Figures 3.6).

3.2. Primary culture of fibroblasts

The protocol of primary culture of fibroblasts was optimised by conducting a series of pilot experiments that made use of published experience.  

3.2.1. Specimens:

Eight tissue samples were collected from uterine specimens of abdominal hysterectomy performed for benign indications on premenopausal Caucasian women with no POP. Five millimetres thick slices of the medial ends of the cardinal ligament were obtained from the part of the cervix above its portio vaginalis (Figure 3.1). The specimens were placed immediately in a cold Hanks’ Balanced Salt Solution, (HBSS, Gibco BRL, Paisley, Scotland, UK) and transferred to the tissue culture laboratory for immediate processing. Collection and processing were done under strictly aseptic conditions.
Figure 3.5

Bland-Altman (SR)-intra-observer
Figure 3.6

Bland-Altman (ECM)-intra-observer
3.2.2. Cell culture:

1- Samples were minced into 1 mm$^3$ pieces, treated with 15 ml of collagenase type I (Gibco BRL, Paisley, Scotland, UK) 1% in HBSS and then incubated at 37°C for 2h with gentle rotation.

2- The cells were washed twice in HBSS and four times in growth medium; Medium 199 Earle’s MOD Salts (Gibco BRL, Paisley, Scotland, UK) supplemented with 15% heat inactivated fetal bovine serum (Gibco BRL, Paisley, Scotland, UK), to which 1% antibiotics/antimycotic solution (10 000 U Penicillin, 10 mg Streptomycin, 25 μg Amphotericin B per ml, Sigma-Aldrich, Dorset, UK) was added. Then, they were spun down at 300xg for 5 min to remove the collagenase.

3- The cellular pellet was resuspended in the growth medium, plated in 25-cm$^2$ tissue culture flasks with Nunclon surface (Nalge Nunc International Corporation, Hereford, UK), and incubated at 37°C in humidified air with 5% CO$_2$. The medium was changed on alternate days and confluent fibroblasts were passaged into a 75-cm$^2$ flask (Nalge Nunc International Corporation, Hereford, UK) using 0.1% trypsin-EDTA (Gibco BRL, Paisley, Scotland, UK) for 10 min at 37°C. The growth of the fibroblasts was monitored with an Inverted Microscope Eclipse TS100-F (Nikon Corporation, Tokyo, Japan). The fibroblasts were then frozen in liquid nitrogen for storage.

3.2.3. Freezing down cells:

1- The fibroblasts were washed once in the growth medium, then trypsinised as mentioned above.

2- They were centrifuged at 300xg for 5 min.
3- The supernatant was aspirated and the fibroblasts were re-suspended in fetal bovine serum, transferred to cryotubes (0.5ml of suspension per tube), and thoroughly mixed with freeze mix (0.5ml per tube).

4- The following freezing regimen was followed:

-20°C for one h, then

-80°C for 24 h, then

-Liquid nitrogen for long-term.

3.2.4. Rising -up cells from liquid nitrogen:

1- The cryotube was removed from the storage and placed into a container of water at 37°C, and when thawed, it was rinsed with 70% IMS to maintain sterile conditions.

2- The cell suspension was added to 5 ml of growth medium and spun at 300xg for 5 min.

3- The supernatant was removed, an appropriate amount of growth medium was added to the pellet and fibroblasts were plated.

4- Fibroblasts from the 4th to 6th passages were used for experimentation.

3.3. Microarray experiments

3.3.1. Fibroblast preparation:

Cells of three different subjects were raised-up from liquid nitrogen, allowed to grow and then seeded (0.5 x 10⁵ cells/well) onto BioFlex® plates; 6-well, flexible, with Collagen-I coated elastomer membrane bottoms (FlexCell International Corporation,
McKeesport, Pennsylvania, USA). Fibroblasts culture was performed following the same protocol described before until 8 plates of sub-confluent fibroblasts were obtained from each subject. They were then serum-starved for 48h, by using 1% charcoal/dextran treated fetal bovine serum (HyClone, Logan, Utah, USA) added to Medium 199 Earle’s MOD Salts supplemented medium, to achieve quiescence. Subsequently, they were cultured with 15% charcoal/dextran treated serum in the same medium. Four plates were exposed to mechanical stretch and the other four were used for the static experiment. There was no treatment added and the only factor tested in this experiment was the effect of stretch versus no stretch. Four cDNA arrays per sample were performed to identify the transcriptional profile.

3.3.2. Stretch regimen:

The Flexercell Stress Unit (FlexCell International Corporation, McKeesport, Pennsylvania, USA) was used for the application of stretch to the cultured cells. The plates were housed within a manifold and subjected to a pre-calibrated computer-generated vacuum. This apparatus allowed strains to be equibiaxial over the majority of the elastomer membrane areas. Release of the vacuum relaxed the membranes and returns them to the condition before straining. Software driven controls of the solenoid valves that regulated the vacuum made it possible to generate various strain amplitudes, durations, and frequencies on cell monolayers. The fibroblasts were exposed to stretch (21%)-relaxation (0%) cycles of 12 hours each at a frequency of 1 Hz for 96h. This regimen was chosen after a series of optimising experiments taking into consideration the capacity of The Flexercell Stress Unit and the durability of the
BioFlex membrane. The identical BioFlex static plates were kept simultaneously in the same incubator at 37°C, and humidified atmosphere with 5% CO₂ (Figure 3.7).

3.3.3. Extraction of total RNA-Trizol method:224

All microfuge tubes (Eppendorf, Axygen Scientific Inc., Union City, USA) and pipette tips (ART tips, Eppendorf, Axygen Scientific Inc., Union City, USA) used were RNAse free.

1- The media was removed and fibroblasts were washed twice for 5 min each with PBS, pH 7.4, pre-warmed to 37°C.

2- One ml of Trizol (Invitrogen Ltd., Paisley, Scotland, UK) was added per well and the plates were incubated at RT for 5 min.

3- The cells were scraped from the wells and the resulting cell lysate of each well was transferred to a microfuge tube.

4- Chloroform (Sigma-Aldrich, Dorset, UK) was added to each tube at 0.2 ml per 1 ml of Trizol and mixed by vigorous shaking for 15 sec. After incubation at RT for 3 min, the samples were centrifuged at 12000xg at 4°C for 15 min.

5- The clear upper aqueous phase (containing RNA) was carefully transferred, without disturbing the lower layer or interface, to a screw cap polypropylene centrifuge tube.

6- To precipitate the RNA, 0.5 ml of isopropranolol (Sigma-Aldrich, Dorset, UK) per 1 ml Trizol was added and mixed with the aqueous phase by repeated inversion. After incubation at RT for 10 min, the samples were centrifuged at 12000xg at 4°C for 10 min.
Figure 3.7

Stretch
7- The supernatant was removed and discarded, and the gelatinous RNA pellets were washed in 1 ml of 75% ethanol (Sigma-Aldrich, Dorset, UK). The tubes were vortexed to resuspend the pellets, and then centrifuged at 7500xg at 4 °C for 5 min.

8- The supernatant was decanted and discarded. The RNA pellets were air-dried for 10 min, and then resuspended in 100 μl of DEPC (diethylene pyrocarbonate)-treated water.

9- Tubes were incubated at 60 °C for 10 min to facilitate dissolution.

10- The quantity (μg/μl) and quality of RNA were determined by measuring RNA absorbance at 260nm (A_{260}) and 280nm (A_{280}) using a UV / Visible Spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK):

- The quantity of total RNA was calculated from the absorbance at 260nm using the following equation:

  \[
  \text{RNA concentration (μg/μl)} = A_{260} \times \text{dilution} \times 40
  \]

- A measure of the purity was given by the A_{260}/A_{280} ratio:
  
  - A ratio of 1.0 for pure RNA
  - A ratio of 1.9 – 2.1 for good RNA

11- The quality and integrity of total RNA were assessed by using Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, CA, USA) and Agilent RNA 6000 Nano Assay (RNA Nano reagent kit guide, part number: G2941-90126).²²⁵ (Figure 3.8)

12- RNA was stored at -80°C until use.
Figure 3.8

RNA
3.3.4. Production of the gene array:

Analysis of global gene expression was carried out using cDNA microarrays containing approximately 4500 expressed sequence tags derived from IMAGE clones obtained from Research Genetics (Huntsville, AL, http://www.resgen.com) or from the MRC Human Gene Mapping Project (Hinxton, Cambridge, http://www.hgmp.mrc.ac.uk). These clones were sequence verified prior to arraying. All of the plasmid inserts were amplified by the use of a universal primer set, and a standard PCR mix (ABGene) employed. The products were all electrophoresed on an agarose gel and the gel images stored for future reference. All PCR products that failed in initial amplification or produced multiple bands were re-amplified using plasmid specific primers. The size of the insert sequence was determined from the agarose gel by comparison with the ladder using MultiAnalyst software (BioRad). After PCR, the reaction products were precipitated and prepared for array using the methods previously described.\textsuperscript{194,226}

\textbf{Printing the array:} Arrays were printed on poly-L-lysine coated slides, and UV cross-linked and blocked prior to use.\textsuperscript{194,226} They were printed using an arrayer built essentially according to the Stanford designs and can be seen at http://www.le.ac.uk/cmht/twg1/array-fp.html.

\textbf{Labelling and hybridisation} (Figure 3.9):\textsuperscript{194,226,227}

\textit{Annealing:}
Figure 3.9

Microarray
1- Priming was achieved using 0.5 μl of oligo dT₂₅ primer (8μg/μl, PNACL, Protein and Nucleic Acid Chemistry Laboratory, Leicester University, UK) with 25 μg of total RNA in 10 μl RNAse-free DEPC-treated water.

2- After denaturation at 70°C for 8 min, annealing was allowed to occur as the temperature fell to 42°C over a period of 30 min.

Labelling:

3- While the tube temperature was maintained at 42°C, the master mix was prepared as follows:

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Volume to add</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAsin (Promega, Southampton, UK)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>5X first strand buffer (GibcoBRL)</td>
<td>4 μl</td>
</tr>
<tr>
<td>0.1M DTT nucleotides (GibcoBRL)</td>
<td>2 μl</td>
</tr>
<tr>
<td>dNTP mix: 20mM dATP, dCTP, dGTP and 8mM dTTP</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Superscript II reverse transcriptase (GibcoBRL)</td>
<td>0.5 μl (100 U)</td>
</tr>
</tbody>
</table>

dNTPs (Pharmacia Biotech, Cambridge, UK) were added to final concentrations of 0.5 mM with the exception of dTTP, which was at 0.2 mM. Superscript II was added just before adding the mastermix to the samples.

4- Cy5 (blue, stretch sample) or Cy3 (pink, control sample) fluor-dUTP (Amersham, Buckinghamshire, UK), 2 μl of 1mM was added to the RNA samples to a final concentration of 0.1 mM and mixed.
5- Then, 7.5 μl of mastermix was added to each tube and mixed by pipetting.

6- Transcription was allowed to proceed for 1h at 42°C before addition of a second 0.5 μl (100 U) of Superscript II, followed by another 1h of incubation at 42°C. A 20.5 μl of water was added to bring the volume to 41 μl.

*Hydrolysis of RNA*

7- Residual RNA was removed from the synthesised cDNA by addition of 1 μl of 0.5M EDTA (to stop the reaction), 1 μl of 10% v/v Sodium Dodedecyl Sulphate (SDS, Sigma-Aldrich, Paisley, Scotland, UK) and 3 μl of 3M NaOH. They were added separately to avoid precipitation of SDS. The tubes were incubated at 70°C for 10 min.

8- The reaction was neutralised by addition of 3 μl of 2M HCl, and buffered to pH 7.5 by addition of 10 μl of 1M Tris/HCl.

9- Subsequently, 1 μl of 4 μg/ml tRNA (Gibco BRL, Paisley, Scotland, UK) was added to the labelled probe to act as a carrier. The total volume at this stage was 60 μl.

*Purification by passage through a Centri-step column (Princeton separations, Adelphia, New Jersey, USA):*

10- A Centri-step column was prepared at least 30 min before use by adding 800 μl of water to the column, and replacing the top caps. The column was stored in this condition at 4°C.

11- The column was inserted in a 2 ml eppendorf tube. The top cap was removed then the bottom one.

12- The tube was spun at 750xg in an eppendorf centrifuge for 2 min.
13- The column was transferred to a new longer collecting tube, and 60 μl of the labelling mixture was pipetted and placed on the centre of the surface of the gel bed.

14- Centrifugation was performed again at 750xg for 6 min to collect the purified probe.

15- Then, 1.0 μl of Poly A (1 μg/μl) was added to one of the pairs of probes and 1.0 μl Cot-1 DNA (10mg/ml, Gibco BRL, Paisley, Scotland, UK) was added to its partner. The purpose of using Cot1 DNA was to avoid having non-specific binding of Alu fragments to the target sequences.

16- The probe was dried down using a speed vacuum.

*Preparation for hybridisation:*

17- One probe was suspended in a total volume of 10.5 μl of the hybridisation buffer and 4.5 μl of 20X SSPE was added. After low speed centrifugation, the content of the other tube was added, followed by further low speed centrifugation. This gave a total probe volume for hybridisation of 15 μl.

18- The entire probe was denatured at 100°C for 2 min followed by cooling down to 42°C.

*Hybridisation:*

19- The array slide was placed over the template slide.
20- The probe was placed on the left of the array. The probe spread out from the edge of the coverslip, and the excess was removed with the pipette.

![Diagram](image.png)

21- A 100μl aliquot of water was placed into both of the wells in the bottom of the hybridisation chamber and the array slide was placed in the chamber. The lid was screwed on tight and placed in a 42°C water bath overnight.

**Washing:**

22- The arrays were washed in 1.0X Saline Sodium Citrate (SSC, 3M sodium chloride and 0.3M sodium citrate) and 0.03% SDS for 10 min by plunging in several times until the coverslip fell off. Then, they were transferred to 0.2X SSC for 5 min and plunged again several times, followed by a final wash in 0.05X SSC for 5 min at RT. At least 200 ml of solution was used per washing.

23- The slides were centrifuged at 700xg for 5 min to remove superfluous fluorescent material, then kept in dark and dry place until scanning.

**3.3.5. Analysis of fluorescence and data processing:**

Pixel intensity for hybridisation was determined using an Axon 4000A scanner and GenePix software (Axon Instruments, Union City, CA) version 3.0.6. Feature sizes
were determined using the inbuilt automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined, and the median fluorescence of these pixel measurements taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default parameters of GenePix 3.0. The raw feature data for each channel were globally centred by reference to the median fluorescence of the whole feature set for that channel. The mean’s.d. of the log2 ratio data was calculated, and used to calculate significance weighing values using the following equation:

\[ f(x) = \frac{1}{1 + \exp[-a(x-b)]} \]

\[ x = \log_2 \text{ratio} \]

\[ a = \frac{5}{(\text{conf 99} - \text{conf 95})} \]

\[ b = \frac{(\text{conf 95} + \text{conf 99})}{2} \]

The signs of the function and confidence values were reversed for the two tails of the distribution. Values between 0.05 and -0.05 were rounded to 0. Significance weighing values greater than 0.92, or less than -0.92 were associated with differential expression ratios falling in the greater than 99% confidence levels in both tails of the distribution. Thus, the middle significance weighing value of 0.5 was associated with the values falling on the 97.5% confidence level and the whole data set was thus normalised to a scale of -1 to 1.

Clustering analysis was performed using the significance weighing. Data from replicate experiments were entered separately. Data for which there was a fluorescence value in
one channel only were treated as a special case. These values were assigned a significance value of +1 or -1 depending on which channel the fluorescence was recorded and only if the intensity of that fluorescence was greater than a multiple of 0.5 to three (dependent on the array quality) multiplied by the average fluorescent intensity for the channel as a whole. Data processing was carried out using Convert Data version 3.2.3a (http://www.le.ac.uk/cnht/twg1/array-fp.html). These values were fed into Cluster 3 (Eisen-http://www-genome.stanford.edu/)\textsuperscript{202} and clustered hierarchically using complete linkage. Prior to viewing, the weighed significance data were converted back into ratio data. The clustered data were displayed using Treeview (http://www.rana.lbl.gov).

3.4 Cytoskeletal proteins studies:

3.4.1. Fibroblasts treatment:

_Preparation for treatment:_

Cells of the eight subjects were raised-up from liquid nitrogen, allowed to grow, and then seeded (0.5 X 10\textsuperscript{5} cells/well) onto BioFlex® plates; 6-well, flexible, with Collagen-I coated elastomer membrane bottoms (FlexCell International Corporation, Pennsylvania, USA). Fibroblast culture was performed following the same protocol described before until two plates of sub-confluent fibroblasts were obtained from each subject. They were then serum-starved for 48h, by using 1% charcoal/dextran treated fetal bovine serum (HyClone, Logan, Utah, USA) added to Medium 199 Earle’s MOD Salts supplemented medium, to achieve quiescence. Subsequently, they were cultured
with 15% charcoal/dextran treated serum in the same medium, and individual wells were subjected to different treatment regimens for 96h.

_Treatment regimens:_

One 6-well BioFlex® plate was subjected to stretch and the other one was used for the static experiment. Each well of both plates was treated daily with one of the following agents: 17β-oestradiol 10⁻⁶M, 17β-oestradiol 10⁻⁹M (Sigma-Aldrich, Dorset, UK), levormeloxifene 10⁻⁶M, levormeloxifene 2 X 10⁻⁷M (Novo Nordisk, Bagsvaerd, Denmark). Controls included 17α-oestradiol 10⁻⁶M (isomeric control, Sigma-Aldrich, Dorset, UK), or ethanol (0.1% v/v, BDH laboratories supplies, Poole, England), which was the solvent used for both oestradiol and levormeloxifene.

**3.4.2. Stretch regimen:**

The same regimen previously described in section 3.3.2 was used.

**3.4.3. Staining for cytoskeletal proteins using fluoroprobe technology:**

The protocol of staining of the cytoskeleton was optimised after reviewing the data sheets of the manufacturer and previously published methods.⁸⁷,¹⁶²,²²⁸-²³¹

1- The fibroblasts were washed twice for 5 min each with PBS, pH 7.4, and then fixed with 3.7% formaldehyde solution (Fisher Chemicals, Loughborough, UK) for 10 min at RT.

2- Then, the fibroblasts were permealised with cold acetone (Fisher Chemicals, Loughborough, UK) at –20°C for 5 min.
3- They were kept overnight in 1% BSA in PBS to reduce non-specific background staining.

4- The fibroblasts were then incubated for 45 min at RT with the fluorescent anti-α-tubulin primary antibody (table 3.2), followed by 2 washes in PBS for 5 min each.

5- This was followed by another 45 min incubation at RT with fluorescent-labelled rabbit anti-mouse IgG antibody (Table 3.3), with subsequent 2 washes in PBS for 5 min each.

6- Nuclear DNA staining was performed for one min using Bisbenzimide Hoechst No 33342 Trihydrochloride B2261 (1:10 000 in PBS, Sigma-Aldrich, Dorset, UK), followed by 2 washes in PBS for 5 min each.

7- Staining for F-actin was performed for 20 min using Texas Red®-X Phalloidin (1:50 in PBS, Molecular Probes, Leiden, The Netherlands), followed by 2 washes in PBS for 5 min each to remove unbound Phalloidin.

8- Finally, the cells were kept in PBS overnight at 4°C to get a clear background.

9- The wells were then separated with a scalpel, attached to slides with nail polish and mounted with coverslips, which were covered with a smear of Prolong® Antifade (Molecular Probes, Leiden, The Netherlands) to extend the useful lives of the staining.

10- The slides were kept between steps in a light-tight box.

11- The slides were stored in a light-tight box at 4°C.

**3.4.4. Microscopic assessment and photography:**

Specimens were examined with a Nikon TE300 inverted fluorescence microscope (Nikon Corporation, Kingston-on-Thames, UK) and photographs were obtained using
Hamamatsu Orca ER black and white digital CCD camera. The fluorescent images were captured using Openlab software (Improvision limited, Coventry, UK). The number of cells with abnormal actin morphology out of a total of 300 cells was quantified by random selection of 10-20 fields per well. Image analysis and count was performed, jointly with a morphologist, while blinded to the type of treatment and to the culture condition.

3.5. Optimisation experiments:

A series of optimisation experiments were conducted before choosing the stretch and treatment regimens.

The two doses of 17β-oestradiol used represented the low physiological and the high pharmacological doses as previously described in the literature, while the two doses of levormeloxifene represented the highest and lowest serum levels in women treated with this drug to prevent osteoporosis (Novo Nordisk' study protocol). A third very high dose (10^{-4}M) of levormeloxifene was used during the optimization phase. Raloxifene, one of the SERMs, was also used at concentration of 10^{-6}M.

Maximum mechanical stretch was used for the longest duration sustained by The Flexercell Stress Unit and the membrane of the BioFlex plates. Time-ranging pilot studies were conducted at 2, 4, 8, 12, 24, 48, 72, 96h, where the duration of mechanical stretch was the only variable, to detect the time when morphological changes appear.
For the 2, 4, 8 and 12h experiments, the stretch was applied for the whole period of experimentation, whereas for the other experiments, the stretch was applied for 12h alternating with the static mode.

In a series of initial experiments, the cells were incubated static for a further four days period after the 96h of stretch. They were examined daily by light microscopy, and Phalloidin staining for F-actin was performed to assess if they were able to recover. Nevertheless, there was a gradual increased rate of cellular death and they became detached from the membrane. By the 4th day, the living cells represented the minority that probably were not well stretched.

### 3.6. The Background of the Statistical Analysis:

In multiple linear regressions, the mean value of the dependent variable is expressed as a linear function of a set of explanatory variables, and for an observed value of the dependent variable, there would be an error term. The classical method applies to the case where the error terms are normally distributed with zero mean and constant variance, but not applicable to the case where the dependent variable is qualitative. However, the concept of a relationship between the distribution of a dependent variable and a number of explanatory variables is just as valid when the dependent variable is qualitative as when it is continuous. The subjects in this research project did not represent a homogeneous group and they differed on a set of explanatory variables. The relationship between the dependent variables (e.g percentage of stained areas for ECM
protein, positive cells for steroid receptors, abnormal actin count) and the explanatory
variables (e.g. prolapse, menopausal status, HRT, stretch) in the different studies was
not linear since a straight-line relationship would imply probabilities outside the
legitimate range of 0 to 1 for some values of the explanatory variables. In order to fit
the relationship into the framework of linear regression it was necessary to apply other
forms of regression. Therefore, the analyses in these studies were based on a series of
generalised linear models (GLM; logistic regression, poisson regression, and gamma
errors regression) with adjustment for clustering using the package “Stata”. GLMs are
regression that can be used to predict responses both for dependent variables with
discrete distributions and for dependent variables, which are non-linearly related to the
predictors. The dependent variable is a proportion, a count, and a positive continuous
variable in logistic regression, poisson regression, and gamma model with log link,
respectively. Although GLMs can be flexible enough to be applied to a wide range of
real-life situation, they retain most of the power of normal linear regression models.
The class of non-normal errors which is used is called the exponential family and
includes binomial (for logistic regression), poisson (for poisson regression), and gamma
(for gamma error regression).234

The link function was used in these studies to model responses (outcomes) since the
dependent variables were non-linearly related to the predictors. It specified a nonlinear
transformation of the predicted values so that the distribution of the predicted values
was one of several special members of the exponential family of distributions
mentioned above.235
All calculations were adjusted for the clustering of results from the same objects. Clustering means that the profiles for subjects were compared, and subjects who were “close” together were classified as being in the same cluster or group. The term “profile” referred to a set of measurements pertaining to a single subject. These might be repeated measurements of a single variable e.g. in the immunohistochemical studies, clustering of measurements was made on different fields within the same slide.\textsuperscript{236}

Another problem arose with multi-contingency tables where there was no obvious outcome variable. The technique of log linear remodelling was used to enable testing the relationship between any two of the variables in the table holding the others constant.\textsuperscript{234}

The package Stata was used for the analysis (StataCorp 2001 Stata Statistical Software Release 7.0 College Station TX: Stata Corporation).

The method used for statistical analysis in every study is detailed in the relevant chapter.
Chapter Four

Changes in extracellular matrix proteins expression in the cardinal ligaments of women with prolapse - A computerised immunohistomorphometric analysis
Introduction

It was suggested that trauma or pathology might lead to altered responses to mechanical stresses by producing changes in the ECM, such as imbalance between collagen synthesis and degradation, or between collagen types, which may predispose women to prolapse. There are also many reported age-related changes that could affect the pelvic floor leading to poor support in older women, including increased elasticity of the connective tissue, gradual denervation, decreased collagen contents, reduced muscle fibre to connective tissue ratio, deterioration of the strength of skeletal muscles, and hypoestrogenic state. These changes contribute to the gradual decline in ligament strength, stiffness and ability to withstand deformation.

Alterations in collagen synthesis and collagen types were causally related to connective tissue disorders, such as urinary incontinence, and it was suggested that the beneficial effect of oestrogen on urethral sphincter function might be mediated by collagen. Also, faulty elastin production was suspected to contribute to the development of POP, and led to the loss of elastic recoil observed with a variety of connective tissue disorders and in the ageing skin. Nevertheless, there is no data in the literature regarding the expression of tenascin in the connective tissue of the pelvic floor of women with prolapse. It was sparsely distributed in normal anterior cruciate ligaments, but the expression increased markedly in the ruptured ligaments, suggestive of involvement in the healing process after ligamentous injury.
**Aim of the study**

Oestrogen deficiency is considered to be the key event that affects ECM, and high incidence of POP is observed in postmenopausal women. This, together with the previously reported low collagen I/collagen III (CI/CIII) ratio with increased laxity of the knee ligaments, down-regulated elastin synthesis by fibroblasts from prolapsed cardinal ligaments, and raised tenascin expression in the ruptured knee ligaments, led to the hypothesis that POP would be associated with alterations in the ECM composition of the cardinal ligaments, and HRT would reverse these changes in postmenopausal women. It was anticipated that these ligaments would have higher expression of collagen III and tenascin, and lower expression of collagen I and elastin. To test this hypothesis, a series of immunohistochemical studies were conducted on paraffin-embedded sections to document the differential expression of four ECM proteins; collagen I, collagen III, elastin and tenascin, in human cardinal ligaments of prolapsed uteri compared to non-prolapsed controls in premenopausal and postmenopausal women, and also in postmenopausal women treated with sequential combined HRT.

**Materials and Methods**

The study population, specimens’ collection technique and IHC protocol are described in chapter 3.
**Image analysis:**

Images of tissue sections were captured using Axioplan microscope (Carl Zeiss, Welwyn, Herts, UK), a colour video camera (Sony CCD/RGB) and KS 300 image analysis programme (Imaging Associates Limited, Thames, Oxfordshire, UK). From each slide, ten randomly selected fields (X 200) were captured. Within each field, the stained area of ECM was outlined, measured, and the percentage of the stained area was processed by the software and transferred to Microsoft excel programme for statistical analysis. Image capture and analysis was performed while blinded to the different groups.

**Statistical analysis:**

P-values and confidence intervals for the percentage of positive areas were analysed by a generalised linear model with gamma errors and a log link, and adjusted for clustering of repeat readings within women. The statistical analysis was performed using the generalised linear model (glm) command of Stata (StataCorp 2001 *Stata Statistical Software Release 7.0* College Station TX: Stata Corporation).

**Results**

**Collagen I:**

The percentage of positive areas for the no prolapse group was 7.1% (95%CI: 2.3%, 21.6%) and 41.9% (95%CI: 26.7%, 65.8%) for pre- and postmenopausal women, respectively. For the prolapse group, the percentage of positive areas was 9.7%
(95%CI: 3.8%,17.4%), 41.3% (95%CI: 29.4%,58.0%) and 23.3% (95%CI: 11.0%,49.1%) in premenopausal, postmenopausal not taking HRT and postmenopausal women on HRT, respectively. These findings demonstrated that collagen I was 4 times higher in postmenopausal women when compared with premenopausal women irrespective of the presence of prolapse. The differences were significant for both no prolapse (P=0.004) and prolapse (P<0.001) groups. There was suggestion of an increase with age in the postmenopausal women, but this does not explain the differences between pre- and postmenopausal women (Figures 4.1 and 4.2a-c).

Collagen III:

The percentage of positive areas for the no prolapse group was 11.5% (95%CI: 7.9%,16.6%) and 17.9% (95%CI: 11.3%,28.6%) for pre- and postmenopausal women, respectively. For the prolapse group, the percentage of positive areas was 48.5% (95%CI: 36.0%,65.2%), 35.8% (95%CI: 26.2%,49.0%) and 12.0% (95%CI: 6.8%,21.2%) in premenopausal, postmenopausal not taking HRT and postmenopausal women on HRT, respectively. In premenopausal women, the percentage of collagen III staining in the prolapse group was significantly higher than that in the no prolapse group (P<0.001). Similarly, in postmenopausal women not taking HRT, the percentage was significantly higher in the prolapse group (P=0.02). Collagen III expression in postmenopausal women was significantly suppressed by HRT (P=0.001). There was no indication of an age effect in any group (Figures 4.3 and 4.2d-f).
Figure 4.1

Collagen I
Figure 4.2

IHC
Figure 4.3

Collagen III
Collagen I/collagen III ratio (CI/CIII ratio):

CI/CIII ratio for the no prolapse group was 1.56 (95%CI: 0.34,7.11) and 2.68 (95%CI: 1.40,5.14) for pre- and postmenopausal women, respectively. For the prolapse group, the ratio was 0.18 (95%CI: 0.08,0.41), 2.16 (95%CI: 0.86,5.43) and 4.24 (95%CI: 1.80,10.00) in premenopausal, postmenopausal not taking HRT and postmenopausal women on HRT, respectively. The ratio was significantly higher in postmenopausal women not taking HRT when compared with premenopausal women (P<0.001). In premenopausal women, the no prolapse group expressed significantly higher ratio than the prolapse group (P=0.02). In postmenopausal women not taking HRT, the ratio was also higher in the no prolapse group compared to the prolapse group, but this was not statistically significant. There was a suggestion of an age effect especially in premenopausal women (Figure 4.4).

Elastin:

The percentage of positive areas for the no prolapse group was 11.7% (95%CI: 7.4%,18.5%) and 17.6% (95%CI: 9.2%,33.5%) for pre- and postmenopausal women, respectively. This was in marked contrast to the consistent lower expression of elastin in the prolapsed ligaments, where the percentage of positive areas was 4.8% (95%CI: 2.3%,10.0%), 2.5% (95%CI: 1.0%,6.4%) and 1.4% (95%CI: 0.7%,3.0%) in premenopausal, postmenopausal not taking HRT and postmenopausal women on HRT, respectively. These findings demonstrated that elastin expression is 3-4 times lower in the prolapsed ligaments irrespective of the menopausal status. In postmenopausal women not on HRT, the percentage of elastin staining in the prolapsed ligaments was
Figure 4.4

Collagen I/III
significantly lower than that in the no prolapse group (P<0.001). Similarly, in
premenopausal women, the percentage was significantly lower in the prolapse group
(P=0.02). There was no indication of an age effect in any group (Figure 4.5 and 4.2g-i).

**Tenascin:**

The percentage of positive areas in the no prolapse group was 0.3% (95%CI: 0.1%,2.4%) and 3.5% (95%CI: 1.6%,7.7%) for pre- and postmenopausal women, respectively. For the prolapse group, the percentage of positive areas was 26.6% (95%CI: 15.6%,45.4%), 26.5% (95%CI: 19.7%,35.6%) and 40.0% (95%CI: 7.3%,58.5%) in premenopausal, postmenopausal not taking HRT and postmenopausal women on HRT, respectively. These findings demonstrated that tenascin expression was significantly higher (more than 10 times) in the prolapse group regardless of the menopausal status (P<0.001). In the no prolapse group, the percentage of tenascin staining was also significantly higher after the menopause (P=0.03). There was no indication of an age effect in any group (Figure 4.6 and 4.2j-l).

**Discussion**

In agreement with the hypothesis, this study showed that the expression of collagen III
and tenascin was significantly increased, while the expression of elastin was
significantly decreased in prolapsed ligaments irrespective of menopausal status. It was
uncertain whether these changes were the cause or the effect of prolapse. Nevertheless,
Figure 4.5

Elastin
Figure 4.6

Tenascin
collagen I was significantly higher in postmenopausal women with no impact for prolapse. Despite this unpredicted finding, CI/CIII ratio was found to be reduced in the prolapsed ligaments because of the higher expression of collagen III. This could have a significant clinical impact since it was generally agreed that a higher CI/CIII ratio in the ligament was indicative of greater strength, whereas a lower ratio was characteristic of tissue laxity.\(^{41-44}\) It was found that collagen I and III have distinctive physical properties and their relative proportions influenced tissue function. While collagen I imparted a great mechanical strength to connective tissues, collagen III appeared to play a role in tissue elasticity and extensibility.\(^{241}\) Moreover, it was reported that the repair of connective tissue, which was damaged by vaginal delivery, involved a change from collagen I to the weaker collagen III.

The role of elastin alteration in POP was unclear until Yamamoto *et al*\(^{80}\) found that steady-state elastin mRNA levels and elastin protein synthesis were significantly down-regulated in quiescent fibroblasts from cardinal ligaments of patients with prolapse compared with controls. These results suggested that the paucity of elastic fibres might contribute to the loss of supportive function in the cardinal ligaments. Adults cannot rebuild the elastic fibre assembly mechanisms, and this lack of regeneration of functional elastic fibres is a major problem, because once damage has occurred, the restoration of normal function is impossible.\(^{242}\)
The pattern of change in tenascin might fit a picture of healing phase of traumatised tissue. Supporting evidence came from the previous reports of raised tenascin expression in the ruptured knee ligaments,\textsuperscript{53} around healing wounds,\textsuperscript{51} and in keloid formation.\textsuperscript{214} The trauma itself might have been initiated by events such as childbirth, and the lack of oestrogen following the menopause resulted in decompensation.

It was assumed that HRT would rectify the damage occurred in the prolapsed ligaments, particularly after the previous reports that oestrogen replacement therapy restored the normal balance of different components of the ECM, and reverted the connective tissue of postmenopausal women towards premenopausal conditions by increasing the proteoglycan/collagen ratio, decreasing collagen cross-linking and reducing collagen content.\textsuperscript{204} Nevertheless, in this study, HRT significantly reduced collagen III in the prolapsed ligaments to levels similar to those of normal ligaments, but did not rectify the alterations in other ECM proteins probably because such regimens lack the myriad steroids manufactured by the premenopausal ovaries.

P-values and confidence intervals for the percentage of positive areas in the cardinal ligament specimens were analysed by a generalized linear model with gamma errors and a log link. This type of regression was used, as previously mentioned, because the distribution of errors of the dependent variable (percentage of stained areas) was highly skewed and the variance was increasing with the mean.\textsuperscript{235} The gamma regression was used particularly because the percentage of stained areas was a positive quantitative variable i.e. it could not be negative.
Given the well-observed interaction between ECM proteins and oestrogen, and the regulation of oestrogen action by ECM proteins, it was speculated that the changes in the ECM composition would alter the expression of the steroid receptors in general and oestrogen receptors in particular in the prolapsed cardinal ligaments. Therefore, evaluating the prevalence of these gonadal steroids receptors in the cardinal ligaments of healthy and prolapsed uteri was considered necessary in order to establish the normal tissue values against which future studies could be based.
Chapter Five

Changes in gonadal steroid receptor expression in the cardinal ligaments of women with prolapse - A computerised immunohistomorphometric analysis
**Introduction**

A number of studies clarified the influence of oestrogen on the ligaments of the knee\textsuperscript{205-207} and hip\textsuperscript{156} joints, but the precise role of oestrogen in the function of pelvic floor, and in the pathogenesis, prevention or treatment of POP is yet to be known. Similarly, the effect of the hormonal milieu in the postmenopausal women on the pelvic ligaments has not been adequately assessed, in spite of the high prevalence of POP in the postmenopausal period.\textsuperscript{4,7,25}

It was recently reported that age- and menopause-related reduction in uterosacral ligament resilience facilitated progression to symptomatic POP if the pelvic floor was previously damaged by vaginal birth.\textsuperscript{18} Furthermore, the clinical development of two SERMs; idoxifene\textsuperscript{168} and levormeloxifene,\textsuperscript{170} used in postmenopausal women to treat and prevent osteoporosis was discontinued because of the observed increased incidence of POP and urinary incontinence. This was strongly suggestive of involvement of the oestrogen receptors in a manner that adversely affects cellular or ECM elements of the connective tissue. However, oestrogen responses might be affected by the relative expression of specific receptor subtypes present in target tissues.\textsuperscript{112}

Gonadal steroids react not only with their cognate receptors, but also they interact with a variable affinity with the other steroid receptors. They influence the rate of production, turnover and expression of the cognate receptor proteins as well as all other nuclear receptors.\textsuperscript{211} Moreover, a high concentration of either the cross-reactive ligand
or the unliganded receptor was speculated to evoke a receptor-specific biological response.\textsuperscript{210,211}

The evaluation of Ki67 staining was widely used to detect the proliferative activity of many tissues including endometrium\textsuperscript{243} and endometriotic lesions,\textsuperscript{244,245} but was not reported previously in the assessment of proliferative activity of pelvic ligaments. However, increased Ki-67-positive cells and observation of mitotic figures were prevalent in the fibroblasts of the degenerating cranial cruciate ligaments of beagle dogs, which attested to the proliferating potential of ligamentous fibroblasts.\textsuperscript{246}

**Aim of the study**

The interaction between ECM proteins and oestrogen, and the regulation of oestrogen action by ECM proteins was well observed.\textsuperscript{208} Moreover, higher expression of the ER\textsubscript{α} and PR was found in the mechanically failed varicose segment of the vein compared to the adjacent healthy segment,\textsuperscript{209} suggesting that steroid receptors could be invoked in the sustenance of structural integrity and functional competence of the ECM. These observations led to the hypothesis that the expression of the oestrogen receptors would be elevated in the prolapsed cardinal ligaments as a compensatory response to the oestrogen deficiency induced alterations in the ECM. It was also hypothesised that PR and AR expression would be elevated because ovarian steroids were known to interact with and influence the expression of each other.\textsuperscript{210} Furthermore, it was assumed that HRT would reverse these changes. To test these hypotheses, another series of
immunohistochemical studies were conducted to investigate the differential expression of gonadal steroids receptors; ERα, ERβ, PR and AR in human cardinal ligaments of prolapsed uteri compared to non-prolapsed controls in premenopausal and postmenopausal women, and in postmenopausal women treated with sequential combined HRT. The density of Ki-67 positive cells, as a surrogate marker of proliferative activity, was also analysed.

**Materials and Methods**

The study population, specimens’ collection technique and IHC protocol are described in chapter 3.

**Image analysis:**

Images of tissue sections were captured using an Axioplan microscope (Carl Zeiss, Welwyn, Herts, UK), a colour video camera (Sony CCD/RIB) and KS 300 image analysis programme (Imaging Associates Limited, Thames, Oxfordshire, UK). From each slide, 15 randomly selected fields (X1000 with oil immersion) were captured. Within each field, the number of positive and negative nuclei was counted using the software, and the data were transferred to the Microsoft Excel programme for statistical analysis. The number of fields per specimen was chosen to satisfy α=0.05 (5% significance) and β=0.80 (80% power). A preliminary evaluation of image analysis data indicated the need for a minimum of 10 specimens per group and 10-15 randomly
selected fields per slide. Image capture and analysis were performed blindly for the different groups.

**Power and Statistical methods:**

Power calculations were based on early data collected on ERα and performed by simulation. Assuming 15 fields per subject, 10 subjects per group and actual mean percent positive cells of 40% in one group and 50% in the other; if the standard deviation (SD) between subjects is 7.5%, then providing that the SD between fields on the same subject is no greater than 5%, the study will have at least 80% power when testing at the 5% level. Calculations were repeated assuming different numbers of fields per subject and it was found that there was little difference in power providing that at least 10 fields per subject were taken. The preliminary work on which the design was based had used 15 fields, and it was decided to stay with that number.

The statistical analysis was performed using Stata (StataCorp 2001 *Stata Statistical Software Release 7.0*, Stata Corporation College Station, TX). Each receptor type was analysed separately. The data on the proportion of positively stained nuclei were analysed using logistic regression with adjustment of the standard errors to allow for clustering of measurements made on different fields within the same slide. Results were presented as percentages of positively stained nuclei and their 95% confidence intervals obtained from the logistic regression. Patient groups were compared using the corresponding Wald tests. Each comparison between patient groups was made with and
without adjustment for age. In general, the age adjustment made no material difference, and unadjusted results were given unless otherwise stated.

## Results

**ERα:**

The average percentage of ERα positive cells for each group, together with their corresponding 95% confidence intervals, is shown in table 5.1 and figure 5.1. In women not on HRT, the ERα expression in prolapsed ligaments was higher than in non-prolapsed ligaments for pre-menopausal ($P=0.09$) and post-menopausal ($P<0.0001$) groups. The test of interaction of prolapse versus menopausal status ($P=0.04$) suggested that the difference in ERα expression with prolapse may be larger in the post-menopausal women (74% versus 32%) than in the pre-menopausal women (60% versus 43%). In the postmenopausal women with prolapse, there was no significant difference between those on HRT and those not on HRT ($P=0.38$). There was no material change in these comparisons when they were adjusted for age (Figure 5.2a-c).

**ERβ:**

The average percentage of ERβ-positive cells for each group, together with their corresponding 95% confidence intervals, is shown in table 5.1 and figure 5.3. The expression of ERβ in the pre-menopausal women with no prolapse was significantly higher ($P=0.02$) than for those with prolapse. However, the expression was nearly
Table 1: The expression of steroid receptors in the cardinal ligament specimens

<table>
<thead>
<tr>
<th></th>
<th>Prolapse</th>
<th>No Prolapse</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PrM</td>
<td>PM</td>
<td>PM/HRT</td>
<td>PrM</td>
<td>PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα</td>
<td>60.4%</td>
<td>74.3%</td>
<td>68.4%</td>
<td>42.5%</td>
<td>31.9%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(42.5%,75.8%)</td>
<td>(67.5%,80.0%)</td>
<td>(55.8%,78.9%)</td>
<td>(32.5%,53.1%)</td>
<td>(21.3%,44.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>32.3%</td>
<td>52.5%</td>
<td>45.0%</td>
<td>67.0%</td>
<td>44.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(15.9%,54.6%)</td>
<td>(36.7%,67.7%)</td>
<td>(30.0%,60.4%)</td>
<td>(48.9%,81.2%)</td>
<td>(25.5%,65.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>27.5%</td>
<td>43.5%</td>
<td>20.7%</td>
<td>5.7%</td>
<td>15.6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12.8%,49.3%)</td>
<td>(28.2%,60.1%)</td>
<td>(9.6%,39.2%)</td>
<td>(2.6%,12.2%)</td>
<td>(7.6%,29.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>40.3%</td>
<td>35.7%</td>
<td>65.4%</td>
<td>20.9%</td>
<td>23.8%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(28.6%,53.3%)</td>
<td>(22.2%,51.9%)</td>
<td>(46.0%,80.7%)</td>
<td>(12.1%,33.6%)</td>
<td>(9.5%,48.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PrM = premenopausal  PM = postmenopausal  PM/HRT = postmenopausal on HRT  AR = androgen receptor
ERα = oestrogen receptor-α  ERβ = oestrogen receptor-β  PR = progesterone receptor
Figure 5.1

ERA
Figure 5.2

IHH
Figure 5.3

ERB
similar in the different groups of postmenopausal women. The test of interaction of prolapse versus menopausal status ($P=0.03$) suggested that the difference in ER $\beta$ expression with prolapse exists only in the premenopausal women (32% versus 67%), but not in postmenopausal women (53% versus 45%). In the postmenopausal women with prolapse, there was no significant difference between those on HRT and those not on HRT ($P=0.52$). There was no material change in these comparisons when they were adjusted for age (Figure 5.2d-f).

**AR:**

The average percentages of AR-positive cells for each group, together with their corresponding 95% confidence intervals, is shown in table 5.1 and figure 5.4. In women not on HRT, the AR expression in prolapsed ligaments was higher than in non-prolapsed ligaments for pre-menopausal ($P=0.004$) and postmenopausal ($P=0.008$) groups. The test of interaction of prolapse versus menopausal status ($P=0.62$) suggested that the difference in AR are related to the prolapse regardless of the menopausal status. Women with prolapse on HRT showed significantly lower expression (21%) of AR in comparison with those not taking HRT (44%). There was no clear evidence for an effect of age (Figure 5.2g-i).

**PR:**

The average percentage of PR-positive cells for each group, together with their corresponding 95% confidence intervals, is shown in table 5.1 and figure 5.5. In women
Figure 5.4

AR
Figure 5.5

PR
not on HRT, the PR expression in prolapsed ligaments was higher than that in the non-prolapsed ligaments for both pre-menopausal ($P=0.03$) and post-menopausal ($P=0.38$) groups. The test of interaction of prolapse versus menopausal status ($P=0.64$) suggested that the difference in PR is related to the prolapse regardless the menopausal status. PR expression in the ligaments of postmenopausal women with prolapse and taking HRT was double the average expression in those not on HRT ($P=0.02$). There was no material change in these comparisons when they were adjusted for age (Figure 5.2j-l).

**Ki-67:**

No Ki-67 expression was detected in any of the ligaments tested.

**Discussion**

The prolapsed ligaments expressed 1.5-2.5 times more ER$\alpha$-positive cells, 3-4 times more AR-positive cells and twice as many PR-positive cells compared with the controls irrespective of the menopausal status. In contrast to the hypothesis, the expression of ER$\beta$-positive cells was twice as high in pre-menopausal women with no prolapse compared with those with prolapse, and no significant difference was found between the post-menopausal groups. The use of HRT in postmenopausal women with prolapse was associated with a statistically significant reduction in the expression of AR and increase in the expression of PR.
These results refer to the fact that the event of prolapse per se was the determinant of finding significantly elevated gonadal steroid receptors expression. Since one of the possibilities for oestrogen’s mode of action could be ascribed to its proliferating effect on fibroblasts, all the sections were stained for Ki-67 antigen to determine the prevalence of such cells. Ki-67 was found not to be expressed in the human cardinal ligaments from all groups, suggesting that the vast majority, if not all, constituting cells were either at G0 or early G1 of the cell cycle where Ki-67 antigen was not expressed. This was a further indication that the detected changes in the steroid receptors expression were not the result of an increased proliferative activity of the fibroblasts, but were a prolapse-related phenomenon.

The finding of decreased expression of ERβ in the prolapsed ligaments contradicted the working hypothesis. Although the ERβ isoform appeared to be closely related to ERα in most tissues, it was also found that some human ERα positive cells lack ERβ and vice versa, suggesting that oestrogen action in some tissues might be mediated via the activation of one subtype rather than the other or both together. This raised the possibility of distinct ERα– and ERβ-dependent transcriptional pathways. While both ERα and ERβ bind oestrogen, the tissue- and ligand-specific functions of ERα appeared to differ from those mediated by ERβ. The tissue responses could be modulated by the extent to which the ER form homodimers (αα and ββ) and heterodimers (αβ), and by the affinity of dimers for a specific ligand. It was previously reported that the differential expression of ER subtypes in the human uterosacral ligaments and vaginal tissues was dependent on the menopausal status. This could explain some
of the tissue-specific effects of SERMs. Further, both ERα and ERβ were detected in the glandular epithelial cells and stromal cells of the endometrium throughout the menstrual cycle; however, ERα expression was more prominent in all cell types in all phases of the cycle. This might suggest that endometrial oestrogenic effects occurred predominantly through ERα. Pathology also modulated the type of ER expression. Experimental injury to the vessel wall increased ERβ expression up to 80-fold in endothelial and smooth muscle cell, while ERα was unaffected.

Another unexpected result from this study was that HRT had only partial ameliorating effects by significantly reducing AR expression in the prolapsed ligaments to levels similar to those of normal ligaments. It was assumed that HRT would reverse all the changes observed in the prolapsed ligaments. This might suggest that oestrogen is not the only factor that plays roles in maintaining or restoring the composition of these ligaments.

Logistic regression was used because the outcome variable (the expression of steroid receptors) is dichotomous or binary i.e. can take only two values - a positive “which could be labelled as 1” or negative “which could be labelled as 0”. A regression equation, which would predict the proportion of positive nuclei or estimate the probability that a certain group would be positive was required. An ordinary linear regression equation was inappropriate because this might predict proportions less than zero or greater than one, which would be meaningless. In other words, the presence or absence of positive staining were nominal data and thus could not be normally
Further, in this study, there were many explanatory variables (prolapse, menopausal status, age, and HRT), therefore the Wald test was used to test the significance of these explanatory variables in the statistical model.

It was essential to investigate the expression of the AR and PR in the cardinal ligaments in addition to the ER because steroids ligands bind not only to their cognate receptors, but also interact, with a variable affinity, with the other steroid receptors. Both oestradiol and progesterone at a 100-fold higher concentration were shown to effectively displace dihydrotestosterone from the AR, and these cross-reacting steroids could also trans-activate the AR-responsive promoter. An in vivo corollary to these results was observed in transgenic mice. Even in the absence of androgen treatment, overexpression of the AR in the female liver in the normal oestrogenic environment caused virilisation of the female liver. In addition, a high concentration of either the cross-reactive ligand or the unliganded receptor was found to evoke a receptor-specific biological response. The extent of binding is demonstrated in figure 5.6. The dissociation constants (Kd) for the reactions \(M + L \rightleftharpoons ML\), is given by 
\[
[M]_{eq}[L]_{eq}/[ML]_{eq},
\]
where \(M\) is free macromolecule, \(L\) is free ligand, and \(ML\) is macromolecule-ligand complex. The unit of Kd is molarity, \(M\). The lower the Kd (i.e. the higher the ML), the tighter the binding and vice versa. Kd varies from about 1 millimolar (mM) for some enzyme-substrate complex, to pico (pM) and femto (fM) molar levels. This illustrates the cross reactivity between various ligands and a given nuclear steroid receptors depending on the amount of the ligand and/or the expressed steroid receptor protein.
Figure 5.6

Binding
The immunohistochemical data of this research project, together with the previous findings of elevated expression of steroid receptors in the mechanically failed vessels wall,\textsuperscript{209,215} and increased tenascin expression both \textit{in vivo}\textsuperscript{51,52,212-214} and \textit{in vitro}\textsuperscript{216} under the effect of mechanical strain, suggested a clear link between the trauma resulting from chronic stretch and the increased expression of gonadal steroid receptors and altered ECM proteins. Therefore, it was decided to explore further the effect of \textit{in vitro} mechanical stretch on primary cultures of fibroblasts derived from the cardinal ligaments for possible linking cellular mechanisms worthy of further studies.
Chapter Six

Changes in transcriptional profile of cardinal ligament fibroblasts in response to mechanical stretch
Introduction

Pelvic ligaments in women in their physiological state are subject to repetitive and sustained deformation forces exerted by gravity and raised intra-abdominal pressure. When these ligaments fail to maintain their function, POP may develop. The importance of cell shape and intra-/extra-cellular tension balance in determination and regulation of cell behaviour is well known and a number of studies on fibroblasts in vitro as well as animal experiments in vivo have demonstrated the capacity of mechanical stretch to modulate cell behaviour through several different signalling pathways. Moreover, stretch induced an adaptation in gene expression profiles. It was found that stretching human dermal fibroblasts led to induction of mechanically-responsive genes involved in connective tissue synthesis and inhibition of matrix degradation, and increased levels of the steady-state mRNA coding for procollagen I, III, and VI, fibronectin, elastin and beta-actin. Nevertheless, the changes in the transcriptional profile of the mechanically stretched cardinal ligaments fibroblasts were not studied before.

Aim of the study

Given the results obtained in the immunohistochemical studies (Chapter 4 and 5), it was hypothesised that expression of a number of genes representing the altered ECM proteins and gonadal steroids receptors proteins would be modified. It was also reasonable to assume that many other genes expression would be changed. These
assumptions were supported by the previous findings of increased expression of steroid receptors in mechanically failed vessels wall,\textsuperscript{209,215} and mechanical strain-induced elevation of tenascin expression \textit{in vivo}\textsuperscript{212-214} and \textit{in vitro}\textsuperscript{216}. The effect of mechanical stretch on primary cultures of fibroblasts derived from the cardinal ligaments was expected to be similar to the effect of chronic stretch associated with prolapse \textit{in vivo}. To test the hypothesis, this study was designed to examine the changes in the transcriptional profile of those fibroblasts derived from cardinal ligament and grown in primary cultures, in response to exposure to mechanical stretch, using cDNA microarray technology.

**Materials and Methods**

Specimens’ collection, primary culture technique, stretch regimen, methodology of total RNA extraction and cDNA microarray protocol are described in chapter 3.

**Statistics:**

The data were normalised, condensed and analysed statistically to a final measure of differential gene expression as described by Zhang & Gant 2004 \textit{et al}\textsuperscript{256} based on the programme accessible at http://www.le.ac.uk/mrctox/microarray-lab.
Results

Signals were rated according to “balanced differential expression”, which took into account labelling efficiency as well as hybridisation background.\textsuperscript{256} Signal intensities are expressed as fold change of Cy5:Cy3 intensities (stretched: static). Positive numbers indicated higher expression and negative numbers indicated $x$-fold lower expression in the stretched model. From 4500 genes only 16 were significantly up-regulated and 18 were down-regulated as a result of stretch compared with static mode ($p<0.05$). Table 6.1 shows a list of these 34 mechano-responsive genes and their currently established functions. Four of these genes, coding for regulation of actin cytoskeleton remodelling and the interaction between the ECM proteins and cytoskeleton, were up-regulated. These include; phosphatidyl inositol-4-phosphate 5-kinase (PIP5K1C), the human signal-induced proliferation associated gene-1 (SIPA-1), TNFRSF1A-associated via death domain (TRADD) and deoxyribonuclease 1-like 1 (DNase 1-L1). Two other genes that control ECM dynamics were affected; transforming growth factor-$\beta$3 (TGF-$\beta$3) was down-regulated and matrix metalloproteinase-20 (MMP-20) was up-regulated.

Discussion

It was hypothesised that the mechanical stretch of the cardinal ligament fibroblasts in primary culture would modify a number of genes representing the altered ECM proteins
Table 6.1: Mechano-responsive genes significantly altered in stretched fibroblasts and identified by cDNA microchip

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Symbol</th>
<th>Unigene mRNA sequence</th>
<th>Main log ratio</th>
<th>p value 2-tail</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actin remodelling regulators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFRSF1A-associated via death domain</td>
<td>TRADD</td>
<td>NM_003789</td>
<td>2.41</td>
<td>0.013</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-phosphate 5-kinase, type I, γ</td>
<td>PIP5K1C</td>
<td>NM_0012398</td>
<td>0.96</td>
<td>0.037</td>
</tr>
<tr>
<td>Signal-induced proliferation-associated gene 1</td>
<td>SIPA1</td>
<td>NM_006747</td>
<td>0.38</td>
<td>0.0008</td>
</tr>
<tr>
<td>Deoxyribonuclease 1-like 1</td>
<td>DNase1L1</td>
<td>NM_006730</td>
<td>0.19</td>
<td>0.049</td>
</tr>
<tr>
<td><strong>ECM/ECM dynamics regulators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 20 (enamelysin)</td>
<td>MMP20</td>
<td>NM_004771</td>
<td>2.25</td>
<td>0.046</td>
</tr>
<tr>
<td>Laminin, beta 2 (laminin S)</td>
<td>LAMB2</td>
<td>NM_002292</td>
<td>0.32</td>
<td>0.036</td>
</tr>
<tr>
<td>Transforming growth factor, β receptor III (betaglycan,300kDa)</td>
<td>TGFB3</td>
<td>NM_003243</td>
<td>-1.54</td>
<td>0.028</td>
</tr>
<tr>
<td><strong>Cell cycle and proliferation regulator</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycoprotein M6A</td>
<td>GPM6A</td>
<td>NM_005277</td>
<td>3.01</td>
<td>0.022</td>
</tr>
<tr>
<td>E74-like factor 3 (ets domain transcription factor,epithelial-specific)</td>
<td>ELF3</td>
<td>NM_004487</td>
<td>0.28</td>
<td>0.009</td>
</tr>
<tr>
<td>Ubiquitin specific protease 13</td>
<td>USP13</td>
<td>NM_003940</td>
<td>0.1</td>
<td>0.028</td>
</tr>
<tr>
<td>Protein phosphatase 1D magnesium-dependent, δ isoform</td>
<td>PPM1D</td>
<td>NM_003620</td>
<td>-0.53</td>
<td>0.029</td>
</tr>
<tr>
<td>Proteasome (prosome, macropain) activator subunit 2 (PA28 β)</td>
<td>PSME2</td>
<td>NM_002818</td>
<td>-0.76</td>
<td>0.047</td>
</tr>
</tbody>
</table>
### Intracellular modulators/signal transducers

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleiomorphic adenoma gene-like 1</td>
<td>PLAGL1</td>
<td>NM_002656</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Surfactant, pulmonary-associated protein C</td>
<td>SFTPC</td>
<td>NM_003018</td>
<td>0.28</td>
<td>0.014</td>
</tr>
<tr>
<td>Electron-transfer-flavoprotein, α polypeptide (glutaric aciduria II)</td>
<td>ETFA</td>
<td>NM_000126</td>
<td>0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>Guanine nucleotide binding protein (G protein), α 11 (Gq class)</td>
<td>GNA11</td>
<td>NM_002067</td>
<td>-0.27</td>
<td>0.046</td>
</tr>
<tr>
<td>Fumarate hydratase</td>
<td>FH</td>
<td>NM_000143</td>
<td>-0.28</td>
<td>0.046</td>
</tr>
<tr>
<td>Glucose phosphate isomerase</td>
<td>GPI</td>
<td>NM_000175</td>
<td>-0.30</td>
<td>0.012</td>
</tr>
<tr>
<td>Mitochondrial elongation factor G2</td>
<td>GFM2</td>
<td>NM_032380</td>
<td>-0.31</td>
<td>0.025</td>
</tr>
<tr>
<td>Glutathione S-transferase A2</td>
<td>GSTA2</td>
<td>NM_000846</td>
<td>-0.35</td>
<td>0.014</td>
</tr>
<tr>
<td>Solute carrier family 22 (organic cation transporter), member 1</td>
<td>SLC22A1</td>
<td>NM_003057</td>
<td>-0.40</td>
<td>0.041</td>
</tr>
<tr>
<td>Tax1 (human T-cell leukaemia virus type I) binding protein 1</td>
<td>TAX1BP1</td>
<td>NM_006024</td>
<td>-0.08</td>
<td>0.031</td>
</tr>
</tbody>
</table>

### Cell growth regulators

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonate 5-lipoxygenase-activating protein</td>
<td>ALOX5AP</td>
<td>NM_001629</td>
<td>-0.09</td>
<td>0.039</td>
</tr>
</tbody>
</table>

### Cell adhesion regulators

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal-induced proliferation-associated gene 1</td>
<td>SIPA1</td>
<td>NM_006747</td>
<td>0.38</td>
<td>0.0008</td>
</tr>
<tr>
<td>CD9 antigen (p24)</td>
<td>CD9</td>
<td>NM_001769</td>
<td>-0.52</td>
<td>0.026</td>
</tr>
</tbody>
</table>

### Cytokines and chemokines

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 1 receptor-like 1</td>
<td>IL1RL1</td>
<td>NM_003856</td>
<td>3.27</td>
<td>0.026</td>
</tr>
<tr>
<td>Protein Description</td>
<td>Accession</td>
<td>Fold Change</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>-----------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 1</td>
<td>CCR1</td>
<td>NM_001295</td>
<td>2.13</td>
<td>0.015</td>
</tr>
<tr>
<td>DNA synthesis/transcription factors/DNA binding proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Replication protein A1, 70kDa</td>
<td>RPA1</td>
<td>NM_002945</td>
<td>-0.21</td>
<td>0.049</td>
</tr>
<tr>
<td>Splicing factor 1</td>
<td>SF1</td>
<td>NM_004630</td>
<td>-0.27</td>
<td>0.024</td>
</tr>
<tr>
<td>Postmeiotic segregation increased 2-like 4</td>
<td>PMS2L4</td>
<td>BC029419</td>
<td>-0.42</td>
<td>0.037</td>
</tr>
<tr>
<td>RER1 homolog (S. cerevisiae)</td>
<td>RER1</td>
<td>NM_007033</td>
<td>-0.61</td>
<td>0.046</td>
</tr>
<tr>
<td>MADS box transcription enhancer factor 2, polypeptide B</td>
<td>MEF2B</td>
<td>NM_005919</td>
<td>-0.80</td>
<td>0.046</td>
</tr>
<tr>
<td>Peptidase activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase A2 (pancreatic)</td>
<td>CPA2</td>
<td>NM_001869</td>
<td>0.08</td>
<td>0.006</td>
</tr>
<tr>
<td>Undetermined molecular function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy specific β-1-glycoprotein 2</td>
<td>PSG2</td>
<td>NM_031246</td>
<td>1.91</td>
<td>0.033</td>
</tr>
<tr>
<td>Sperm associated antigen 11</td>
<td>SPAG11</td>
<td>NM_016512</td>
<td>-0.18</td>
<td>0.022</td>
</tr>
</tbody>
</table>
and gonadal steroids receptors observed in the immunohistochemical studies described in chapter 4 and 5. In spite of the sound assumption, the stretch model used in this experiment failed to confirm the immunohistochemical findings. This lack of consistency could be attributed to the duration, amplitude, periodicity and/or frequency of the stretch used in this model. The aim was to exert maximum traction on the fibroblasts to mimic the characteristic chronic excessive stretch of the prolapsed cardinal ligaments; therefore, the stretch model was developed such that maximum stretch for a maximum duration was exerted to the limits endured by the elastomer membrane. The amplitude of 21% stretch in 12h cycles for total duration of 96h proved to be the limits of the apparatus beyond which these membranes started to leak. In view of the fact that cDNA microchip is a comparative microarray experiment, which is rather variable at the individual gene level, replication was considered to minimise the chance of getting false positive results. Three biological replicates were performed, where RNA was extracted from three different subjects, to obtain averages of independent data and to validate generalisation of the conclusions. For each of these extractions, four technical replicates were performed to assist in reducing the variability. The cells were serum-starved, for 48 hours before stretching them, to achieve quiescence. It was previously shown that after 24 hours of serum starvation, more than 70% of the cells were in the same resting phase of the cell cycle (G₀ phase) during which cells down-regulated the proliferation rate and protein synthesis. This ensured that a maximum difference was obtained after mechanical stretch.
By adopting the fold change in statistically significantly (p<0.05) modulated genes, 34 mechano-responsive genes were identified. In exploring the function of these genes through the literature, four of the up-regulated genes were found to code for the regulation of actin cytoskeleton remodelling and its interaction with the ECM proteins. These include; PIP5K1C, SIPA-1, TNFRSF1A-TRADD and DNase 1-L1. Further, two genes were found to control ECM dynamics, including TGF-β3, which was down-regulated, and MMP-20, which was up-regulated. The lack of verification method, such as reverse transcriptase (RT)-PCR, was acknowledged as week point in this study, which was imposed by the limited resources.

The transmission of signals from the plasma membrane to the underlying actin was found to be fundamental for its remodelling. Integrin-ligand binding initiated integrin-mediated intracellular signals in response to cell-matrix adhesion, which via a cascade of events resulted in remodelling of the actin cytoskeleton.91,92 Several other molecules were also shown to transmit signals from plasma membranes to the underlying actin cytoskeleton, such as the family of small GTPases (Rho, Rac and Cdc42). The protein product of PIP5K1C gene was activated to produce PIP2 under the influence of these small GTPases. In turn, PIP2 (i) enhanced actin assembly,95,101,102 (ii) assisted actin interaction with the plasma membrane by causing dissociation of G-actin from actin-monomer-binding proteins,103 (iii) modulated the activity of many actin regulatory proteins, such as profilin, gelsolin, vilin, filamin, vinculin and talin that orchestrate actin polymerisation by the addition of actin monomers at the rapidly growing end (+) of actin nuclei,95 (iv) acted with Cdc42 as cofactors for the activation of two families of
proteins, WASP and ERM, both of which were found to link the plasma membrane with cytoskeletal remodelling. WASP was reported to activate a complex of proteins called Arp2/3 (actin-related protein), which could initiate the formation of actin branches from pre-existing filaments. ERM proteins, like WASP proteins, could bind to PIP₂, which seemed to enhance the ability of these proteins to interact with plasma membrane proteins, and (v) inhibited actin depolymerising effect of DNase 1-L1 and cofelin. The reduction in PIP₂ levels after either injection of a specific antibody or overexpression of synaptojanin, an enzyme that hydrolys PIP₂, interfered with the organisation of stress fibres. In contrast, high concentrations of PIP₂ were accompanied by extensive actin polymerisation and stimulated the formation of microvilli.

Nonetheless, the other three up-regulated genes; DNase 1-L1, TRADD, and SIPA-1 were known to have negative impact on actin cytoskeleton remodelling. The products of these three genes exerted their effects through 3 different pathways. The binding of DNase 1-L1 protein with monomeric (G) actin prevented actin polymerisation, and this binary complex was rendered more stable and more effective when coflin bound to a different site on the G actin molecule forming a “cofilin-actin-DNase I” ternary complex. Cofilin belongs to the actin depolymerising factor (ADF)/cofilin family, which inhibited actin assembly and controlled the turnover rate of actin filaments. This inhibition of actin polymerisation was countered by inactivating phosphorylation signals from both the Rho and Rac pathways. Further, PIP₂ inhibited Cofilin/ADF binding to G actin and dissociated DNase 1-L1 from its complex with G actin thus illustrating the closely linked functions of the products of these altered genes.
Stimulation of TNFR-1 induced an overall decrease in F-actin and inhibited Cdc42-dependent filopodia formation in macrophages, possibly by activation of ADF/cofilin. When the TNF-induced decrease in F-actin was inhibited using either receptor mutants or the compound D609, a distinct type of actin reorganisation involving an increase in F-actin was observed in response to TNF. SIPA-1 overexpression was shown in vitro to inhibit Rap1-dependent cell-matrix adhesion, which was found to be the initial step in the chain of events leading to actin cytoskeleton remodelling.

This apparently conflicting pattern of gene expression in response to mechanical stretch was not surprising because it might well be that the cells tried to compensate for the damage induced by the proteins of DNase 1-L1, TRADD and SIPA-1 genes by overexpressing PIP5K1C, given the fact that the integrity of actin filaments is fundamental for cell survival. It could also be attributed to the long duration and the high amplitude of stretch used in this study. Supporting evidence came from the work of Skutek et al who found that the duration of stretch modulated apoptosis. Application of mechanical stretch (with frequency of 1 Hz and amplitude of 5%) to human tendon fibroblasts increased apoptosis rate after 15 min of stretch by induction of “stress-activated protein kinase pathway”, but the longer stretch period of 60 min was not associated with such increase in apoptosis rate due to development of stress tolerance, possibly by heat-shock protein 72-mediated suppression of the activated “stress-activated protein kinase pathway”.
A down-regulated TGF-β3 and an up-regulated MMP-20, known to control ECM dynamics, were also identified by this experiment. Border et al.\textsuperscript{271} reported that the concept of “matrix-synthesising” fibroblasts was supported by the identification of a variety of tension-inducible growth factors that were known to stimulate matrix deposition in processes like wound healing, scarring, and fibrotic diseases. Similarly, Kessler et al.\textsuperscript{183} found that stretching human dermal fibroblasts led to a synthetic fibroblast phenotype characterised by induction of connective tissue synthesis while simultaneously inhibiting matrix degradation as suggested by up-regulation of TGF-β1 and β-3 and connective tissue growth factor. In contrast, this study found TGF-β3 to be down regulated and MMP-20 to be up regulated, which implied that the regulation of genes controlling ECM dynamics was related to the amplitude and duration of stretch. The other possibility was that the behaviour of cardinal ligament fibroblasts could be different from that of dermal fibroblasts. The role of mechanical stretch in controlling the homeostasis of the ECM was also supported by finding that it significantly suppressed the production of TIMP-1, but not MMP-1 and -2, in scleral fibroblasts of the human eyes.\textsuperscript{180} Although MMP-20 was originally cloned from tooth tissues,\textsuperscript{272} it was also found recently in tongue carcinoma cells, where it hydrolysed collagen IV, laminin 1 and 5, and TN-C.\textsuperscript{273}

A possible simplified model for regulation of actin cytoskeleton remodelling following cell adhesion is shown in figure 6.1.

The findings of this study was a major turning point in this research project, and led to the hypothesis that mechanical strain e.g. in cases of increased intra-abdominal pressure
Figure 6.1

Regulation of actin assembly
**Figure 6.1**: Possible simplified model for regulation of actin cytoskeleton remodelling following cell-matrix adhesion. β-integrin binds to focal adhesion proteins, such as talin or α-actinin. This induces activation of Rho family of small GTPases, which in turn influence the activity of PIP5K1C and stimulate PIP₂ production. Both Cdc42 and PIP₂ function as cofactors for the activation of WASP, which in turn, activates Arp2/3 complex inducing actin cytoskeleton remodelling. TNFR-1 reduces F-actin possibly by inhibiting Cdc42 and/or activating ADF/Cofilin. The later is inhibited by both small GTPases and PIP5K1C. SIPA-1 negatively regulates Rap1, which is required for cell adhesion induced by ECM. DNase 1-L1 prevents actin polymerisation and gets inhibited by PIP₂.

ADF/Cofilin = Actin Depolymerising Factor/Cofilin  
Arp2/3 = Actin-Related Protein  
DNase 1-L1 = Deoxyribonuclease 1-like 1  
ECM = Extracellular matrix  
PIP₂ = Phosphatidyl inositol 4,5 bisphosphate  
PIP5K1C = Phosphatidyl Inositol-4-Phosphate 5-Kinase  
SIPA-1 = Signal-induced proliferation associated gene-1  
TNFR-1 = Tumour Necrosis Factor Receptor-1  
WASP = Wilcott-Aldrich Syndrome Protein
or SERMs could cause POP by destroying the cytoskeleton of the fibroblasts. The work was, therefore, extended to study the effect of mechanical stretch and levormeloxifene on the morphology of the α-tubulin and F-actin of those fibroblasts grown in primary cultures. Further, the partial ameliorating effects of oestrogen therapy on the quality of the prolapsed tissues observed in the immunohistochemical studies led to explore the effect of 17β-oestradiol on the cytoskeleton morphology of fibroblasts using the same model.
Chapter Seven

Changes in cytoskeleton morphology of cardinal ligaments fibroblasts in response to stretch and levormeloxifene
**Introduction**

The nature of the molecular response of pelvic supportive structures to either oestrogen or SERMs remains unclear in spite of the speculated fundamental role of oestrogen in maintaining the integrity of the pelvic floor muscles and ligaments.

cDNA microarray, as previously mentioned in chapter 6, identified four up-regulated genes coding for regulation of actin remodelling in the stretched cardinal ligament fibroblasts in primary culture. In addition, a number of *in vitro* studies showed that mechanical stretch modified fibroblasts proliferation,\textsuperscript{174,176-178} and changed fibroblasts morphology, which appeared elongated, bipolar, and oriented along the lines of tension, while they appeared stellate with short processes in relaxed collagen lattices.\textsuperscript{176} These findings suggested a detrimental effect for mechanical stretch on the cytoskeleton morphology, an area that was not explored before.

**Aim of the study**

The findings of the cDNA microarray study (Chapter 6) that revealed alterations in the expression of some genes involved in the regulation of actin cytoskeleton remodelling, combined with the previously reported mechanical stretch-induced changes in fibroblasts morphology,\textsuperscript{176} led to the hypothesis that the application of mechanical stretch to primary cultures of fibroblasts derived from the cardinal ligament would exhibit changes in cytoskeletal configuration. Assuming that this model of cell stretch
was representing an *in vitro* equivalent to the pressure sustained by the cardinal ligaments *in vivo*, then it would be plausible to hypothesise that SERMs induced POP in healthy asymptomatic women by the same mechanism, and that oestrogen would prevent and/or reverse this effect and maintain the integrity of the fibroblasts. To test these hypotheses, the effect of mechanical stretch, levormeloxifene, and $17\beta$-oestradiol on the morphology of the cytoskeleton ($\alpha$-tubulin and F-actin) of those fibroblasts grown in primary cultures was investigated using the fluoreprobes technology. The possibility whether stretch suppressed proliferation of fibroblasts was raised when a lower cell count was observed in the stretched wells when compared to the static ones. Therefore, it was decided to assess the proliferation index in the stretch and static models, and in response to different treatments.

**Materials and Methods**

Specimens’ collection, primary culture technique, treatment regimen, stretch regimen, protocol of staining for cytoskeletal proteins and details of microscopic assessment and photography are described in chapter 3.

**Assessment of proliferation Index:**

This experiment was designed to examine the effect of $17\beta$-oestradiol $10^{-9}$M, Levormeloxifene $10^{-6}$M, ethanol or no treatment on proliferation of cultured fibroblasts in the stretch and static modes. The same mechanical stretch protocol described in chapter 3 was used for 96h. Each treatment was applied to three wells in the stretch
mode and another three in the static mode. Using the haemocytometer, four counts were performed by two independent observers who were blinded both to the type of treatment and to the culture condition. The given result of each individual treatment represents the average of the number of cells in the three wells counted four times each.

**Losartan experiment:**

Another experiment was designed to examine the effect of losartan (AT\textsubscript{1}-receptor antagonist) on fibroblasts in primary culture. Losartan was found to significantly attenuate the effect of stretch on vascular smooth muscle cells.

The fibroblasts were either treated with levormeloxifene \(10^{-6}\)M or not treated with any drug, and were exposed to either stretch or static mode in the presence or absence of losartan \(10^{-5}\)M for 2 h.

**Statistics:**

Confidence intervals and p-values for the percentage of actin abnormalities were obtained by gamma error regression with a log link function, while the confidence intervals and p-values for the cell counts from the proliferation experiment were obtained by Poisson regression with a log link function. All calculations were adjusted for the clustering of results from the same objects. Calculations were performed using the glm command in Stata (StataCorp 2001. Stata Statistical Software Release 7.0. College Station Texas: Stata Corporation).
Results

Assessment of cytoskeleton morphology:

Normal morphology of F-actin: In normal fibroblasts, F-actin appeared as well defined, brightly red fluorescent, discrete filaments. The stress fibres ran from one edge of the cell to the other (Figure 7.1-1,2), continued into very long filopodia (Figure 7.1-3), and sometimes crossed one another (Figure 7.1-4).

Abnormalities in F-actin: A number of changes in actin phenotype, imposed by stretch and levormeloxifene treatment (10^{-6}M and 2x10^{-7}M), were observed. The most frequent was the partial disintegration of the filaments into small spots and micro-aggregates especially at the cell periphery (Figure 7.1-5), but when these microfilaments aggregates coalesced, the cell shape was markedly altered (Figure 7.1-6). Another prominent feature in many cells was the diminished number of stress fibres (Figure 7.1-7,8,9), while an alteration of cell shape into arborised configuration was distinctly seen in few cells (Figure 7.1-10,11). Some cells showed that the microfilaments were extensively dissolved and their organisation into stress fibres was lost. A remarkable dissolution of the microfilaments was often found in the central region, whereas fine filaments preserved at the periphery of the cells (Figure 7.1-12,13).

Tubulin and nuclear DNA staining: No morphological differences were observed in the microtubules (Figure 7.1-14,16) or the nuclei (Figure 7.1-15,16) regardless of the type of treatment or exposure to stretch.
Figure 7.1

Cytoskeleton
**Figure 7.1 (1-16):** Normal and abnormal patterns of cytoskeleton morphology.

(1,2) Normal stress fibres run from one edge of the cell to the other

(3) Normal stress fibres continue into very long filopodia

(4) Normal stress fibres cross one another

(5) Partial disintegration of the stretched filaments into small spots and microaggregates

(6) Microaggregates coalescence and alteration of the cell shape under the effect of both stretch and levormeloxifene

(7) Diminished number of stress fibres under the effect of levormeloxifene

(8) Diminished number of stress fibres under the effect of stretch

(9) Diminished number of stress fibres under the effect of both stretch and levormeloxifene

(10) Alteration of cell shape into arborised configuration under the effect of stretch

(11) Alteration of cell shape into arborised configuration under the effect of levormeloxifene

(12) Dissolution of the stretched microfilaments

(13) Dissolution of the levormeloxifene-treated microfilaments

(14,16) Normal microtubules

(15,16) Normal nuclei
The effect of levormeloxifene $10^{-4} M$:

Using this high concentration, the actin filaments were extensively dissolved after 2h of treatment both in the stretch and in static models. In many cells, clustering of microfilaments occurred. The experiments were repeated three times and observed by two independent observers (Figure 7.2).

The effect of losartan:

The microfilaments disappeared completely after 2h of treatment both in the stretch and in static models. The experiments were repeated three times and observed by two independent observers.

The effect of raloxifene $10^{-6} M$:

There were no morphological changes observed in the actin filaments or microtubules when static fibroblasts in primary cultures were treated with raloxifene.

Cell count for F-actin abnormalities:

The percentage of cells with abnormal actin configuration was significantly higher in fibroblasts subjected to stretch when compared with the static model ($P<0.0001$). The statistical analysis did not show evidence of interaction ($p=0.76$) i.e. the effect of stretch appeared to be the same with the six different treatments (4-8 times higher with an average of $\geq 5.9$ times, and 95% CI of 3.71 to 9.48). Since the effect of stretch was the same irrespective of treatment, separate comparisons for individual treatments were not required; therefore, the comparison was averaged over the whole experiment. The
Figure 7.2

L4
frequencies of actin abnormalities were also significantly higher with both concentrations of levormeloxifene (P<0.0001) when compared with ethanol. This was consistent irrespective of stretch. The effect of levormeloxifene 10⁻⁶ and 2 X 10⁻⁷M, with pooling the stretch and no stretch results, was ≥ 2.5 times (95% CI: 1.5 to 4.2) and ≥ 2.2 times (95% CI: 1.5 to 3.3) the effect of ethanol, respectively. Both these confidence intervals were significantly different from 1. There were no significant differences between either concentration of 17β-oestradiol, 17α- oestradiol and ethanol vehicle (Table 1a and 1b). Figure 7.3 shows the estimated ratios and 95% confidence intervals for the percentage of cells with abnormal actin morphology. It represents the comparisons between the effect of stretch (regardless of treatment) v no stretch, and also the comparison between the effect of individual treatment versus ethanol.

**Proliferation index:**

The estimated means and 95% confidence intervals for the cell counts from the Poisson regression are shown in table 7.2. Stretch resulted in 23% (P=0.04) and 28% (P<0.001) reduction in cell count when treated with ethanol or no additive, respectively. However, after 96h of treatment, 17β-oestradiol 10⁻⁹M was associated with 39% (P=0.02) increase in cell count when the cells were stretched. There was no significant change with levormeloxifene.
Table 7.1a: The estimated means for the percentage of cells with abnormal actin morphology.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stretch</th>
<th>No stretch</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-oestradiol 10^{-6}M</td>
<td>12.63</td>
<td>1.75</td>
</tr>
<tr>
<td>17β-oestradiol 10^{-9}M</td>
<td>11.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Levormeloxifene 10^{-6}M</td>
<td>27.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Levormeloxifene 2 x 10^{-7}M</td>
<td>23.88</td>
<td>5.13</td>
</tr>
<tr>
<td>17α-oestradiol 10^{-6}M</td>
<td>10.88</td>
<td>1.13</td>
</tr>
<tr>
<td>Ethanol (control)</td>
<td>9.75</td>
<td>2.50</td>
</tr>
</tbody>
</table>

-The data represent the average findings of 8 experiments.

Table 7.1b: The estimated ratios and 95% confidence intervals for the percentages of cells with abnormal actin morphology.*

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>P Value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stretch</td>
<td>5.93</td>
<td>0.0001</td>
<td>3.71,9.48</td>
</tr>
<tr>
<td>17β-oestradiol 10^{-6}M</td>
<td>0.94</td>
<td>0.54</td>
<td>0.78,1.16</td>
</tr>
<tr>
<td>17β-oestradiol 10^{-9}M</td>
<td>0.83</td>
<td>0.32</td>
<td>0.58,1.20</td>
</tr>
<tr>
<td>Levormeloxifene 10^{-6}M</td>
<td>2.55</td>
<td>0.0001</td>
<td>1.54,4.22</td>
</tr>
<tr>
<td>Levormeloxifene 2 x 10^{-7}M</td>
<td>2.21</td>
<td>0.0001</td>
<td>1.50,3.26</td>
</tr>
<tr>
<td>17α-oestradiol 10^{-6}M</td>
<td>0.71</td>
<td>0.09</td>
<td>0.48,1.06</td>
</tr>
</tbody>
</table>

*The data represent the comparison between the effect of stretch (regardless of treatment) versus no stretch, and the comparison between the effect of individual treatment versus ethanol.
Figure 7.3

Actin count
Table 7.2: The effect of stretch on fibroblast proliferation after 96h of treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Number x 10^4 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Static</td>
</tr>
<tr>
<td>17β-oestradiol 10^-9M</td>
<td>8.9 (7.1,11.3)</td>
</tr>
<tr>
<td>Levormeloxifene 10^-6M</td>
<td>11.3 (8.7,11.9)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10.9 (8.9,13.4)</td>
</tr>
<tr>
<td>No treatment</td>
<td>12 (11.2,12.8)</td>
</tr>
</tbody>
</table>
Discussion

This novel study documented for the first time the effect of mechanical stretch, levormeloxifene and oestradiol on the cytoskeleton morphology of fibroblasts in primary cultures. In agreement with the hypothesis, the mechanical stretch caused significant disruption in actin morphology; including partial disintegration of actin filaments into small spots, reduction in the number of stress fibres, alteration of the shape of many cells into arborised configuration and dissolution of the microfilaments. Moreover, Levormeloxifene caused significant changes in actin morphology of fibroblasts in the static culture similar to those induced by stretch. Nonetheless, the use of oestradiol did not reverse the process or protect the cells from the effect of stretch, and the hypothesis of the possible protective or ameliorating role for oestrogen could not be proved by the model used. In contrast to F-actin, neither mechanical stretch nor levormeloxifene caused morphological changes in the arrangement and distribution of α-tubulin, which was consistent with the findings in cell lines derived from human periodontal ligaments. Similar actin collapse was reported in murine hepatocyte treated with Clostridium difficile toxins using confocal microscopy. Similar to the experiment described in chapter 6, cells were serum-starved for 48 hours before stretching them to achieve quiescence and ensure maximum differences between different treatments.

The significant reduction in cell proliferation observed in this study in response to stretch could be attributed to the up-regulation of TRADD and DNase 1-L1 (see
Apoptosis was known to be induced by death receptors of the TNF family and Dnase 1-L1 protein expression was increased prior to the induction of apoptosis. In counterbalance, the raised expression of SIPA-1 could act as a compensatory mechanism for the death signal; since it was previously reported that SIPA-1 protein induced the lymphocyte proliferation by interacting with either or both Rap1 and Ran, members of small GTPases family. It was clear that the stretch-mediated effect on cell division was influenced by the amplitude, frequency, periodicity, and duration of the applied stretch as well as the cell type. Zeichen et al found time-dependent response on stretching human patellar tendon fibroblasts. Proliferation was induced by application of cyclic mechanical stretch (with 1 Hz frequency and 5% amplitude) for 15 or 60 min, and inhibited when it was applied for 30 min. Furthermore, it was reported that cyclic stretching of fetal rat lung cells (with 1 Hz frequency and 5% amplitude for 48h) significantly increased cell proliferation and reduced cell-doubling time. Nevertheless, low magnitude (0.45%) cyclic stretching for 72h did not affect the proliferation of scleral fibroblasts.

As previously mentioned in chapter 3, the cells which were incubated for four days after the 96h of stretch during the optimisation phase of this experiment were examined daily by light microscopy, and Phalloidin staining for F-actin was performed to assess if they were to recover. However, there was a gradual increased rate of cellular death and they became detached off the membrane. By the 4th day, the living cells represented the minority that probably were not well stretched. This was the major constraint, which disallowed studying the changes in nascent protein production, and directed the
work towards exploring the morphological changes in cytoskeleton based on the findings of the microarray.

Losartan was used assuming that it could have an ameliorating effect on the actin cytoskeleton affected by stretch and levormeloxifene. In contrast, it caused total destruction of actin filaments after 2h of treatment. Previous studies showed that it attenuated the stretch-induced rise in collagen α1 mRNA and fibronectin protein synthesis by vascular smooth muscle cells. However, its effect on the cytoskeleton of vascular smooth muscle cells was not assessed in these studies and it could have been the same as in fibroblasts.

To take into account the skewed data, the degree of variability between the individual wells treated with the same treatment, and the fact that the outcome variable (the percentage of cells showing F-actin abnormalities) could not have negative values, the confidence intervals and p-value for the percentage of actin abnormalities was obtained by gamma errors regression with a log link function. Nevertheless, in the cell proliferation experiment, the confidence intervals and p-values for the cell counts were obtained by Poisson regression. This was ideal because there were many time intervals but the numbers of events per interval was small, so that the assumptions of multiple regression (i.e. normal distribution and uniform variance) did not apply.
Phalloidin was used to investigate actin morphology because its specific tight binding to F-actin had facilitated morphological studies.279 No cross-reaction with other cytoskeleton proteins or components associated with the cytoskeleton was known.280 Phalloidin was not found to bind to G-actin, and produced no detectable non-specific staining of other cellular components.
Chapter Eight

General Discussion
Our knowledge about the mechanism of POP has advanced very little over the past 100 years. Understanding the normal anatomy and physiology of the pelvic supporting structures as well as the pathophysiology of support failure are vital to develop adequate preventative and treatment modalities.

The characteristic changes in the prolapsed ligaments

As a result of assuming the erect posture, the female pelvic ligaments are subjected to the effects of gravity and the mechanical stress of the intra-abdominal pressure, and as such are exposed to a form of continuous trauma that intact ligaments can cope with. When these ligaments fail to sustain their mechanical competence they yield to the effect of intra-abdominal pressure and prolapse ensues. The mechanical competence of a ligament is based on co-operative structural arrangement of the constituting fibroblasts as well as the quality and quantity of ECM proteins. Reviewing the literature provided the following leads:

**First:** The menopause is widely accepted to play a role in the pathogenesis of POP, and oestrogen deficiency is considered to be the key event that affects ECM. Oestrogen-related molecular and structural changes in hip and knee ligaments, and altered collagen I and III, elastin, and tenascin expression in the failing ligaments were well-documented.
Second: the observed regulation of oestrogen action by ECM proteins\textsuperscript{208} and the high expression of the ER$\alpha$ and PR in the mechanically failed varicose vein segments\textsuperscript{209} suggested that steroid receptors could be invoked in the sustenance of structural integrity and functional competence of the ECM. Further, the significant increase in the incidence of POP as a result of the use of levormeloxifene and idoxifene pointed to the involvement of oestrogen receptors in the pathogenesis of proplase.\textsuperscript{170,168}

These observations led to the hypothesis that prolapsed cardinal ligaments would have higher expression of collagen III and tenascin, and lower expression of collagen I and elastin. It was also predicted that the expression of ER$\alpha$, ER$\beta$, PR and AR would be elevated in the prolapsed cardinal ligaments as a compensatory response to the oestrogen deficiency-induced alterations in the ECM. Further, it was assumed that HRT would reverse these changes in postmenopausal women. These hypotheses were tested in a series of immunohistochemical studies, which were designed to answer the following questions:

1. Does the state of prolapse have characteristic features that distinguish it from non-prolapsed ligaments? To test this, non-prolapsed ligaments were included as control.

2. Does oestrogen deficiency impart characteristic features on pelvic ligaments? To test this, cardinal ligament specimens from pre-and postmenopausal women were included.
3. Does oestrogen replacement reverse the changes caused by the menopause? To test this, one of the study groups exclusively included samples of ligaments obtained from postmenopausal women receiving HRT.

4. Could the proliferating effect of oestrogen on fibroblasts be responsible for any identified differences in the attributes of cardinal ligament? To test this, all sections were stained for Ki-67 antigen.

The attributes of the prolapsed ligaments

The findings of the immunohistochemical studies were consistent with the hypothesis except for collagen I and ERβ. Collagen I expression was significantly higher in postmenopausal women with no impact for prolapse, while ERβ expression was higher in the non-prolapsed ligaments. In spite of the unpredicted behaviour of collagen I, CI/CIII ratio was lower in the prolapsed ligaments because of the significant increase in collagen III, suggesting that the ability of the ligament to withstand loading is dependent on the balance between different types of collagen rather than the absolute amount of an individual type. The excessive stretching of the prolapsed ligaments could be attributed to increased tissue laxity caused by the high expression of collagen III, in addition to the loss of elastic recoil due to paucity of elastic fibres. The increased tenascin expression might be a compensatory mechanism to prevent further stretching. Interestingly, the increased expression of collagen III, tenasin, ERα, AR and PR in the prolapsed ligaments occurred irrespective of the menopausal status, indicating that these changes were prolapse-related phenomena. The lack of expression of the proliferative marker Ki-67 antigen in the human cardinal ligaments from all groups
provided further evidence that the event of prolapse per se, rather than proliferation of the constituting fibroblasts, was the determinant of the observed changes.

The high expression of gonadal steroid receptors in the prolapsed ligaments could be a compensatory mechanism by the starved ligaments, in response to postmenopausal oestrogen deficiency, in order to increase the binding capacity with the small amount of ligands available. However, there was no oestrogen deficiency state to explain the elevated expression of these receptors in the prolapsed ligaments of premenopausal women. The finding of lower expression of ERβ in the prolapsed cardinal ligaments did not support the working hypothesis. It was assumed that ERβ expression would also be increased for two reasons. First, expression levels of the ERβ isoform was found to be closely related to ERα in most tissues. Second, it was anticipated that the trauma associated with prolapse would induce ERβ expression, based on the previous findings of 80-fold increase in ERβ expression in endothelial and smooth muscle cell of the vessel wall by experimental injury. This unpredicted finding could be attributed to the fact that some human ERα positive cells lack ERβ and vice versa, suggesting that oestrogen action in some tissues might be mediated via the activation of one subtype rather than the other or both together. This was obvious in the glandular epithelial cells and stromal cells of the endometrium where both isoforms were detected throughout the menstrual cycle, but ERα expression was more prominent in all cell types in all phases of the cycle.
The role of hormone replacement therapy

Another unanticipated observation made in this study was that HRT had only partial ameliorating effects by significantly reducing collagen III and AR expression in the prolapsed ligaments to levels similar to those of normal ligaments. The hypothesis that HRT would reverse the full spectrum of changes observed in the prolapsed ligaments was sound for many reasons. First, the previous reports showed that oestrogen replacement therapy restored the normal balance of different components of the ECM and reverted the connective tissue of postmenopausal women towards premenopausal conditions by increasing the proteoglycan/collagen ratio, decreasing collagen cross-linking and reducing collagen content. Second, oestrogen treatment of wounds in experimental animals and in human altered the nature of the extra-cellular matrix. Third, the prevalence of oestrogen receptors in pelvic ligaments made it plausible to assume a pivotal role for oestrogen in maintaining the integrity of these structures by controlling the morphology and key molecular events in fibroblasts development. Lastly, it seemed to be conventionally accepted, at least in some early cases of prolapse, that the use of oestrogen might alleviate the symptoms of the prolapse. Nevertheless, there could be other reasons that made HRT fall short of rectifying the alterations in the prolapsed ligaments and limited the beneficial effects. Oestrogen might not be the only factor that plays a role in maintaining or restoring the composition of these ligaments, and a “supplementary” drug might be required along with oestrogen therapy to strengthen tissue architecture and enhance healing. Additionally, HRT regimens are probably lacking the myriad steroids manufactured by the premenopausal ovaries. It is equally important to consider the fact that progestogens, whose only use is to oppose
the proliferative effect of oestrogen on the endometrium, are known to counter some of
the beneficial effect of oestrogen on different body systems. It would have been unsafe
and unethical to use unopposed oestrogen in these women for the sole purpose of
research.

The possible relation between the changes in ECM and steroid receptors
As already mentioned, oestrogen influences the composition of ECM.\textsuperscript{25,162-166} On the
other hand, the regulation of oestrogen action by ECM proteins is also well
documented, and \textit{in vitro} studies cited the inhibition of ER-mediated transcriptional
activity, including cell proliferation and PR expression, when MCF-7 and T47D breast
cancer cell lines were grown on laminin, but oestrogen therapy induced DNA synthesis
and restored other ER-mediated transcriptional activity including PR expression when
these cancer cell lines were grown on collagen 1 or fibronectin.\textsuperscript{208} Furthermore, the
laminin effect seemed to be more specific against liganded ER-mediated actions, since
the mitogenic responses of these cell lines to insulin-like growth factor-1 (IGF-1) or
epidermal growth factor (EGF) were unaffected.\textsuperscript{208}

It might well be that oestrogen deficiency in postmenopausal women or lack of
response to it in premenopausal women had led to the changes in the ECM composition
found in this study, which in turn triggered the overexpression of steroid receptors in
the prolapsed cardinal ligaments.
The effect of stretch on the transcriptional profile of fibroblasts

The immunohistochemical data of this research project made it plausible to hypothesise that the expression of a number of genes coding for proteins involved in maintaining the cellular and extracellular integrity, and those representing the altered ECM proteins and gonadal steroids receptors would be modified in the prolapsed ligaments. Given the fact that increases in intra-abdominal pressure e.g. heavy lifting, chronic constipation and chronic cough might precipitate POP, the stretch model was adopted for the time since studies were lacking on an experimental model with regard to investigating the cellular events occurring with POP. It was assumed that the effect of long duration mechanical stretch on cardinal ligament fibroblasts in primary culture grown on a collagen matrix would be similar to the effect of in vivo chronic stretch associated with prolapse. cDNA microarray technology was used to investigate the changes in the transcriptional profile of these cells in the stretch versus static models.

cDNA microchip experiments demonstrated significant changes in six genes involved in the regulation of actin cytoskeleton remodelling, cell matrix interactions and the control of ECM dynamics. Nevertheless, these experiments failed to document alterations in gene expression commensurate with the findings obtained from immunohistochemistry on ECM proteins and gonadal steroid receptors. It was surprising not to find at least an up-regulated tenascin gene, which was significantly elevated in the prolapsed cardinal ligaments as shown in the immunohistochemical studies of this research project. Tenascin expression was found to be controlled by
mechanical forces and it increased in tissues undergoing active remodelling, such as in epithelial growth in organogenesis,\textsuperscript{212} bone formation,\textsuperscript{213} wound healing,\textsuperscript{51} keloid formation,\textsuperscript{214} and around tumours.\textsuperscript{52} Similarly, \textit{in vitro} mechanical stretch induced tenascin-C mRNA and protein,\textsuperscript{216} and activated tenascin-C promoter.\textsuperscript{58} In addition, it was assumed that increased tenasin might contribute to the loss of tissue architecture characteristic of POP by its recognised interference with cell-matrix adhesion,\textsuperscript{63,282} and suppression of Rho family\textsuperscript{283} with subsequent disruption of stress fibre organisation. In this respect, it could actually be classified as an adhesion-modulating ECM protein since it was found to modulate adhesion of cells to fibronectin and interfere with the function of integrin in cell adhesion and intracellular signalling pathways.\textsuperscript{63}

There might be a number of reasons for the lack of consistency between the immunohistochemical and cDNA microarray findings. First, the duration, amplitude or frequency of the stretch used in this model. Although, a number of cycle durations and frequencies were tried during the optimisation phase, it would have been an unrealistic demand on the resources to do multiple cDNA microchips to make so many comparisons. Taking into consideration the chronic nature of the prolapse, the stretch model was developed such that maximum stretch for a maximum duration was exerted to the limits endured by the elastomer membrane bottoms. Second, some technical constraints in cDNA microchip could have led to these findings, and a different profile might have been obtained by the Affymetrix technique, which was not available in the department. Third, there must be differences between the stretch model of fibroblasts and the \textit{in vivo} conditions, which include those related to deficiencies of the natural
growth mediators and cytokines, the application of arbitrary load cycles, and the isolation of the fibroblasts from a potentially favourable in vivo environment.

Lastly, extracting the RNA directly from the prolapsed cardinal ligament could have produced results that mimic those of immunohistochemistry; however, in view of the marked inter-subject variability, a large number of samples would be needed to conduct such investigation. It was also not feasible to obtain large enough tissue specimens to collect adequate amount of mRNA per sample.

Despite using biological and technical replication to minimise the chance of getting false positive results and the robust statistical evaluation of significant changes, the use of a verification method, such as reverse transcriptase (RT)-PCR would have further strengthened the confidence in the cDNA microarray data. However, the limited resources did impose not to perform such an experiment.

Morphological changes of cytoskeleton

Given the up-regulation of genes involved in the control of actin cytoskeleton remodelling in the microarray analyses, it was hypothesised that the application of mechanical stretch to primary cultures of fibroblasts derived from the cardinal ligaments would disrupt the cytoskeletal configuration. Further, SERMs might have induced POP in healthy asymptomatic women by the same mechanism, and oestrogen
would prevent and/or reverse the effect of stretch and SERMs, and maintain the integrity of the fibroblasts. To test these hypotheses, the effect of mechanical stretch, levormeloxifene, and 17β-oestradiol on the morphology of the cytoskeleton (α-tubulin and F-actin) of those fibroblasts grown in primary cultures was investigated using the fluroprobe technology.

In agreement with the hypothesis, the mechanical stretch, and levormeloxifene in the static culture significantly disrupted actin configuration. Nonetheless, the hypothesis of the possible protective or an ameliorating role for oestrogen could not be proved in the model used. In contrast to the effects on F-actin, neither mechanical stretch nor levormeloxifene caused morphological changes in the arrangement and distribution of α-tubulin. The mechanistic basis for SERMs-induced POP remained to be elucidated. It was previously alleged that some SERMs caused tissue oedema, thus increasing the risk for prolapse by increasing the weight of the uterus. This did not seem reasonable since enlarged uteri with fibroids did not necessarily prolapse. Many speculations were made as a result of the current study regarding the possible mechanism of action of levormeloxifene. First, the effect on actin could be due to an ineffective binding with oestrogen receptor, however, the study failed to show any ameliorating effects for oestradiol on actin morphology. Second, the mechanism by which levormeloxifene and/or stretch reduced the number and caused abnormalities of F-actin fibres could, at least in part, be explained by depolymerisation into G actin, which was not detectable by the stain used in this experiment. Third, levormeloxifene and/or stretch could have disturbed the stability of the actin, which was found to be largely related to the specific
cross links of its constituting proteins. Although oestradiol did not reverse the process or protect the cells from the effect of stretch, it significantly increased the proliferation of the stretched fibroblasts, suggesting a role in the healing process.

In this study, a cell culture model was deployed, which provided thinly spread fibroblasts. It could have been of value to look for cytoskeletal abnormalities in tissue sections of prolapsed and healthy ligaments, using electron micrographs, but since only relatively short segments of the filaments and microtubules could be included in the ultra-thin sections, this line of investigation could not be perused. Moreover, prolonged exposure to conventional fixatives used in electron micrographs was found to destroy actin filaments.

**Concluding remarks**

The state of a “prolapsed ligament” was associated with changes in the ECM proteins suggestive of a weaker structure as evidenced by heightened expression of collagen III and an ongoing tissue trauma marked by increased tenascin expression. The current data suggested the involvement of gonadal steroid receptors with the mechanical failure of prolapsed ligaments. Cell-matrix adhesion, which is crucial for maintaining normal tissue architecture and function, could be influenced by these modifications of the composition of the ECM. The cytoskeleton assembly is a reflection of the cell interaction with its environment and its integrity promotes cell survival. Since cell shape largely depends on the cell-matrix interaction, it is not surprising that ECM can
have decisive functions in regulating genes expression, particularly those involved in the control of cell architecture. The damage observed in F-actin assembly with stretch and levormeloxifene could be an indication of disturbed cellular communication with ECM. The detrimental effect of levormeloxifene on the integrity of pelvic ligaments could be due to either an adverse modulation of the oestrogen receptors action or direct influence on the structural integrity of the fibroblasts, and subsequently the surrounding ECM constituents, or both.

The stimulatory effect of oestradiol on fibroblast proliferation in vitro may prove to be the basis for the role of oestrogen in tissue recovery and maintaining the integrity of the ligaments. Nevertheless, it only partially restored the normal tissue composition of the ligament in vivo, and did not protect the actin assembly from the destructive effects of the mechanical stretch in vitro. Other steroids, as well as cytokines and growth factors, produced by the pre-menopausal ovary should be explored for their contributory role to the integrity of pelvic ligaments.

The use of the model described in this thesis represents an exciting starting point to contribute to the understanding of the pathogenesis of POP, as well as the role of oestrogen in the prevention and treatment. Further optimisation is required and more favourable culture beds needs to be explored. The use of confocal microscopy in exploring the changes in cytoskeleton in healthy and prolapsed ligament can be developed to substantiate the findings in vitro and to further validate the usefulness of the stretch model used in this project.
Appendix A: Author’s Publications

Based on the research contained in this thesis

Peer reviewed publications:


Submitted for publications:


Journal abstracts:


**Conference abstracts:**

1- **Poster Presentation:** Steroids receptors expression in the cardinal ligaments of postmenopausal women with and without utero-vaginal prolapse: An immunohistochemical study  
   **Meeting:** 10th World Congress on the Menopause  
   **Venue:** ICC, Berlin, Germany  
   **Date:** 10-14th June 2002  

2- **Poster Presentation:** An immunohistochemical study of oestrogen and androgen receptors expression in the ligamentous support of the uterus in postmenopausal women with and without urogenital prolapse  
   **Meeting:** British Menopause Society Annual Conference  
   **Venue:** Brighton Conference Centre, UK  
   **Date:** 4-5th July 2002  

3- **Oral Presentation:** Changes in the cytoskeleton of cardinal ligaments fibroblasts – Effect of stretch & levormeloxifene  
   **Meeting:** 30th British Congress of Obstetrics and Gynaecology  
   **Venue:** SECC, Glasgow, Scotland, UK  
   **Date:** 7-9th July 2004
Appendix B: Future Research

It is well recorded that stretch-mediated effect on cell division, and probably also on transcriptional profile, is influenced by the amplitude, periodicity, and duration of the stretch applied. If enough resources were available, the microarray work would have been extended to determine the pattern of change of transcriptosome on day-to-day basis employing different amplitudes and durations. Further, microarray studies on RNA extracted from levormeloxifene and oestradiol treated cells, with and without stretch, would have been performed to determine the changes in the expression of genes encoding for actin and/or its binding proteins. Assessment of nascent protein assessment using S35 methionine for the products of those mechano-responsive genes identified should have been done, but the observed cell death after the period of stretch was the limiting factor. Nonetheless, modifying the stretch model may make it possible in the future.

Given the significant alterations in ECM proteins expression in the prolapsed cardinal ligaments, and the overexpression of MMP-20 and laminin genes on stretching the fibroblasts, it would be a valuable addition to the work to investigate the expression of other ECM proteins such as fibronectin and some members of MMPs and TIMPs families in the cardinal ligaments. Similarly, studying the differential expression of various members of the integrin family in the cardinal ligaments should be considered in the future work in this field.
Finding similar morphological changes in the actin cytoskeleton in the cardinal ligaments specimens would have confirmed the findings, and would have approved the mechanical stretch model as a representative of the *in vivo* prolapse of these ligaments. Unfortunately, the three-dimensional organisation of the cytoskeleton could not be satisfactorily studied in electron micrographs because of many technical constraints. Improvement of the technology in the future will make such study the logical next step.

All the experiments have been done on specimens/fibroblasts derived from cardinal ligaments, being the principal supportive structure of the uterus. However, performing similar investigations on the other pelvic floor supportive structures is an area that needs to be visited in the future development of this model.

The list of possible topics, taking the findings of the present study a step further, is very long indeed, and the above represents only a small sample. However, with the continuing increase in the numbers of elderly women in the British society with a substantial burden on the health services in terms of morbidity associated with POP, this field of research will remain a focus of interest, with a great potential for further development.
Appendix C: Solutions and Reagents

Bisbenzimide Hoechst No 33342 Trihydrochloride B2261 (Sigma-Aldrich, Dorset, UK)

To prepare a stock solution (stable for 6 months at -20°C):

- 1mg powder
- 1ml dH2O

To prepare a working solution (immediately before use):

- 1μl stock solution
- 10ml PBS

Collagenase

It was made up of mixture of:

- 0.25g collagenase type 1 (GibcoBRL, Paisley, Scotland)
- 100ml Hank's Balanced Salt w/o phenol red (HBSS, GibcoBRL, Paisley, Scotland)
- 1ml Antibiotic/Antimycotic (Penicillin 10 000, 10mg Streptomycin, 25μg Amphotericin B/ml, Sigma-Aldrich, Dorset, UK)

Citrate buffer

- 9ml of 0.1M citric acid
- 41ml sodium citrate
- 450ml dH2O
**dNTP mix** (Pharmacia Biotech, Cambridge, UK)

- 25μl dGTP (20 mM final)
- 25μl dCTP (20 mM final)
- 25μl dATP (20 mM final)
- 10μl dTTP (8 mM final)
- 40μl dH₂O

Final volume: 125μl

**Ethylenediaminetetraacetic acid (EDTA)**

To prepare 1M solution:

- 146.1g EDTA (Fisher Chemicals, Loughborough, UK)
- 400ml dH₂O
- NaOH pellets (until pH is 8.0 and the mixture becomes clear)
- Add dH₂O up to 500ml

**Freeze Mix**

- 15.2ml Medium 199 Earle's MOD salts (GibcoBRL, Paisley, Scotland, UK)
- 4ml Dimethyl Sulfoxide, DMSO (Sigma-Aldrich, Dorset, UK)
- 0.8ml Hepes (1M) (GibcoBRL, Paisley, Scotland, UK)

**Hybridisation buffer**

- 1 ml deionised formamide (Sigma-Aldrich, Dorset, UK)
- 100μl 50X Denhardts
- 200µl dH2O
- 100µl 10% Sodium Dodecyl Sulphate (SDS, Sigma-Aldrich, Dorset, UK)

**Medium 199 Earle's MOD salts** (250ml, 2X, GibcoBRL, Paisley, Scotland), with L-Glutamine, without phenol red. The following were added:

- 170ml sterile dH2O
- 75ml heat inactivated fetal bovine serum (Sigma-Aldrich, Dorset, UK), or 75ml Charcoal/dextran treated fetal bovine serum (HyClone, Logan, Utah, USA)
- 5ml Antibiotic/Antimycotic (Penicillin 10 000, 10mg Streptomycin, 25µg Amphotericin B/ml, Sigma-Aldrich, Dorset, UK)

**Phosphate buffered sulphate (PBS)**

- 0.2g KCl
- 8g NaCl
- 1.15g Na2HPO4
- 0.2g KH2PO4
- 800ml dH2O

Adjust pH to 7.4 with HCl

Add dH2O up to 1000ml

**PBS-Tween 20** (0.05%, Sigma-Aldrich, Dorset, UK)

- 1ml of Polyoxyethylene-Sorbitan Monolaurate
- 2L PBS
**SSPE (20X, 3M NaCl, 1mM NaH$_2$PO$_4$, 20mM EDTA)**

- 43.8g NaCl
- 200ml dH$_2$O
- 5ml 1M NaH$_2$PO$_4$
- 20ml 0.5M EDTA

Then,

Adjust pH to 7.4 using NaOH

Add dH$_2$O up to 250 ml

**Texas Red®-X Phalloidin** (Molecular Probes, Leiden, The Netherlands)

-To prepare a stock solution (stable for long-term at -20 °C):

1.5ml methanol to be added to the Texas Red®-X Phalloidin tube

-To prepare a working solution (immediately before use):

- 1μl stock solution
- 49μl PBS

**Trisma Buffered Saline (TBS)**

- 60.75g Tris
- 87.66g NaCL
- 500ml dH$_2$O

Adjust pH to 7.6 with HCl

Add dH$_2$O up to 1000ml
References:


15. Goh JT. Biomechanical and biochemical assessments for pelvic organ prolapse. 

1993;36(4):897-909.


18. Reay Jones NH, Healy JC, King LJ, Saini S, Shousha S, Allen-Mersh TG. Pelvic 
connective tissue resilience decreases with vaginal delivery, menopause and 

19. Jorgensen S, Hein HO, Gyntelberg F. Heavy lifting at work and risk of genital 
prolapse and herniated lumbar disc in assistant nurses. *Occup Med (Lond)* 

pathogenic factor in uterovaginal prolapse and urinary stress incontinence. *Br J 

21. Sharf B, Zilberman A, Sharf M. Electromyogram of pelvic floor muscles in genital 

22. Landon C, Smith ARB, Croft C, Trowbridge E. Biomechanical properties of 
connective tissue in women with stress incontinence of urine. *Neurourol 
Urodynam* 1989;8:369-370.

of pelvic floor muscles in patients with pelvic relaxation and genuine stress 

prolapse and stress incontinence of urine. A histological and histochemical 

metabolism in postmenopausal women with genuine stress incontinence. *Bjog* 


Figure 1.1: Diagram of measurements taken for pelvic organ prolapse quantification.

**Aa point**: 3 cm from the hymen on the anterior vaginal wall.

**Ba point**: most distal point of upper anterior vagina from hymen when compared to point A.

**C point**: lowest point of cervix.

**Bp point**: most distal point of upper posterior vagina from hymen when compared to point A.

**Ap point**: 3 cm from the hymen on the posterior vaginal wall.

**D**: posterior fornix.

**pb**: perineal body.

**gh**: genital hiatus.

**tvl**: total vaginal length.
Figure 1.2 Principal components of connective tissues

**CONNECTIVE TISSUES**

**CELLS**
- Fibroblasts: fibrous connective tissue
- Chondrocytes: cartilage
- Osteoblasts and osteocytes: bone

**EXTRACELLULAR MATRIX**

**FIBRES**
- Collagen
- Elastic

**PROTEOGLYCANS**
- Aggrecan
- Versican
- Biglycan
- Decorin
- Perlecan

**GLYCOPROTEINS**
- Fibronectin
- Tenascin
- Link Protein
- Fibromodulin
- Thrombospondin
- Osteopontin

**TISSUE FLUID**
**Figure 1.3 Structure of Human ERα and ERβ**

<table>
<thead>
<tr>
<th>ERα</th>
<th>A/B (N-terminal)</th>
<th>C</th>
<th>D</th>
<th>E/F (C-terminal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transcription activation domain (AF-1)</td>
<td>DNA-binding domain (DBD)</td>
<td>Hinge domain</td>
<td>Ligand-binding domain (LBD) &amp; transcription activation domain (AF-2)</td>
</tr>
</tbody>
</table>

**Homology**
- 30%
- 97%
- 30%
- 59%
Figure 3.1: Diagram illustrating the site of cardinal ligament sampling

The medial end of the cardinal ligament
Figure 3.2 Haematoxylin and Eosin (H&E) staining of cardinal ligament (magnification X 200). (a) prolapsed ligament, (b) Non-prolapsed ligament. There is no discernable difference between prolapsed ligaments and controls.
**Figure 3.3:** Bland-Altman plot of inter-observer error in counting steroid receptors in randomly selected 12 slides. It shows the difference between the two means against the average of two means.
Figure 3.4: Bland-Altman plot of inter-observer error in measuring extracellular matrix in randomly selected 5 slides. It shows the difference between the two means against the average of two means.
Figure 3.5: Bland-Altman plot of intra-observer error in counting steroid receptors in randomly selected 6 slides. It shows the difference between the two observations against the average of the two observations.
Figure 3.6: Bland-Altman plot of intra-observer error in measuring extracellular matrix in randomly selected 6 slides. It shows the difference between the two observations against the average of the two observations.
**Mechanical Stretch:**

- **Amplitude:** cycles of 21% stretch & 0% relaxation
- **Periodicity:** 12 hours each
- **Frequency:** 1 Hz
- **Duration:** 4 days

- Both BioFlex plates → same incubator: 37°C + humidified atmosphere + 5% CO₂

**Figure 3.7:** Stretch Regimen Used in All Experiments
**Figure 3.8:** good quality RNA as assessed by using Agilent 2100 bioanalyser

*In cDNA microarray experiments, 3 biological replicates for each 4 technical replicates were performed*
Figure 3.9: Summary of cDNA Microarray Procedures (n=12)

0.5μl of oligo dT25 primer (8μg/μl) + 25μg of total RNA in 10μl RNase-free DEPC-treated water

70°C for 8 min → then 42°C

Add Cy3 (pink, control sample) or Cy5 (blue, stretch sample) fluor-dUTP (2μl of 1mM)

1h at 42°C

Add 7.5 μl of master-mix to each tube:
RNAsin (0.5μl) + 5X buffer (4μl) + 0.1M DTT nucleotides (2μl) + dNTP mix (0.5μl) + Superscript II Reverse transcriptase (0.5μl)

1h at 42°C

Add another Superscript II (0.5μl)

10 min at 70°C

Add 0.5 M EDTA (μl), 10% SDS (μl) and 3M NaOH (3μl)

1h at 42°C

Add tRNA (1μl, 4μg/ml)

purification column

Add Poly A (1.0μl, 1μg/μl) to one of the of probes & Add Cot-1 DNA (1.0μl, 10mg/ml) to its partner

dried vacuum

Suspend one probe in 10.5μl of the hybridisation buffer → Add 4.5μl of 20X SSPE → Centrifugation → Add the content of the other tube → Centrifugation

100°C for 2 min → cooling down to 42°C

Place the array slide over the template slide → Place 15 μl probe on the left of the array → Probe spreads out from the edge of the coverslip

42°C water bath overnight

Wash arrays in 1X SSC & 0.03% SDS for 10 min → 0.2X SSC for 5 min → 0.05X SSC for 5 min

Centrifugation at 2750 rpm for 5 min

Keep in dark and dry place until scanning
Figure 4.1: The percentage expression (Mean and 95% CI) of Collagen I in tissue sections of the cardinal ligaments. It is significantly higher in postmenopausal not taking HRT when compared with premenopausal women, both in prolapse and no prolapse groups. HRT = hormone replacement therapy   PrM = premenopausal   PM = postmenopausal   P = prolapse   NP = no prolapse    ** = p<0.001
Figure 4.2: Immunohistochemical staining of the cardinal ligaments sections for extracellular matrix proteins (magnification X 200). HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse  NP = no prolapse
Figure 4.3: The percentage expression (Mean and 95% CI) of Collagen III in tissue sections of the cardinal ligaments. It is significantly higher in the prolapse group when compared to no prolapse group, both in premenopausal and postmenopausal women. The expression in postmenopausal women is significantly suppressed by HRT. HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse  NP = no prolapse  * = p<0.05  ** = p<0.001
Figure 4.4: Collagen I/III ratio is significantly higher in the prolapse group in postmenopausal women not taking HRT when compared with premenopausal women. It is also significantly higher in the premenopausal women in the no prolapse group when compared with the prolapse group. HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse  NP = no prolapse  * = p<0.05  ** = p<0.001
Figure 4.5: The percentage expression (Mean and 95% CI) of elastin in tissue sections of the cardinal ligaments. It is significantly higher in the no prolapse group irrespective of the menopausal status. HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse  NP = no prolapse  * = p<0.05  ** = p<0.001
Figure 4.6: The percentage expression (Mean and 95% CI) of tenascin-C in tissue sections of the cardinal ligaments. It is significantly higher in the prolapse group regardless of the menopausal status.

HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse
NP = no prolapse  ** = p<0.001
**Figure 5.1:** The percentage expression (Mean and 95% CI) of ERα in tissue sections of the cardinal ligaments. It is significantly higher in the prolapse group when compared with the no prolapse group in postmenopausal women not taking HRT. ERα = Oestrogen receptor-α HRT = hormone replacement therapy PrM = premenopausal PM = postmenopausal P = prolapse NP = no prolapse ** = \( P < 0.001 \)
Figure 5.2: Immunohistochemical staining of the cardinal ligaments sections for gonadal steroids receptors (X 1000). PrM = premenopausal  PM = postmenopausal  PM/HRT = postmenopausal on HRT  NP = no prolapse  P = prolapse
Figure 5.3: The percentage expression (Mean and 95% CI) of ERβ in tissue sections of the cardinal ligaments. It is significantly higher in the no prolapse group when compared with the prolapse group in the premenopausal women. ERβ = Oestrogen receptor-β  HRT = hormone replacement therapy  PrM = premenopausal  PM = postmenopausal  P = prolapse  NP = no prolapse  *= P<0.05
Figure 5.4: The percentage expression (Mean and 95% CI) of AR in tissue sections of the cardinal ligaments. It is significantly higher in the prolapse group when compared with the no prolapse group in both pre- and postmenopausal women not taking HRT. The expression was significantly lower in the in postmenopausal women taking HRT when compared with non-HRT takers. AR = androgen receptor HRT = hormone replacement therapy PrM = premenopausal PM = postmenopausal P = prolapse NP = no prolapse * = P<0.05 ** = P<0.001
Figure 5.5: The percentage expression (Mean and 95% CI) of PR in tissue sections of the cardinal ligaments. It is significantly higher in the prolapse group when compared with the no prolapse group in the premenopausal women. The expression was significantly higher in the postmenopausal women taking HRT when compared with non-HRT takers. PR: progesterone receptor   HRT = hormone replacement therapy   PrM = premenopausal   PM = postmenopausal   P = prolapse   NP = no prolapse   * = P<0.05
Figure 5.6 The extent of ML binding. When Kd gets progressively lower, binding will increase, especially as L gets greater. Kd = The dissociation constants, $M_0 =$ free macromolecule, $L_0 =$ free ligand, ML = macromolecule-ligand complex.
Figure 6.1: Possible simplified model for regulation of actin cytoskeleton remodelling following cell-matrix adhesion
Figure 7.1 (1-16): Normal and abnormal patterns of cytoskeleton morphology
Figure 7.2 (1-6): Effect of levormeloxifene 1 x 10⁻⁴ M on actin filaments. Dissolution and clustering occurred within 2h.
Effect of Stretch and Levormeloxifene on Actin Morphology

Figure 7.3: The estimated ratios and 95% confidence intervals for the percentages of cells with abnormal actin morphology. The plot represents the comparison between the effect of stretch (regardless of treatment) versus no stretch, and the comparison between the effect of individual treatment versus ethanol. The data represent the average findings of 8 experiments. S = Stretch NS = No Stretch E = Oestradiol L = Levormeloxifene *p=0.0001