Connective tissue changes in patients with rectal prolapse

Edward A. E. Smyth

Lincoln College, University of Oxford

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Contents

Abstract

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Rectal prolapse is a common and distressing condition in which the rectum either protrudes externally or telescopes insides itself (intussceception) resulting in evacuatory dysfunction. It has traditionally been viewed as being a disorder of multiparous women who sustain pelvic floor injury during childbirth. However, this explanation fails to account for the occurrence of the condition in nulliparous females, males and children.

This thesis seeks to study the relationship between connective tissue changes and the development of rectal prolapse.

Participants with rectal prolapse were prospectively recruited to the study (n=105) control tissue was taken from individuals undergoing colonic cancer surgery (n=12). Individuals were assessed for their connective tissue status and grouped according to parity and gender. Analyses focussed on presenting demographics and surgical outcomes in patients with respect to connective tissue status, changes in the extracellular matrix in relation to prolapse, systemic changes in connective tissue components.

It was found that a proportion of patients have heritable predisposition to altered connective tissue biology in that they had benign joint hypermobility syndrome. These patients typically presented younger and had higher surgical re-intervention rates than those with normal connective tissue status. The extracellular matrix had a number of changes in association with the condition, collagen types 1 and 3 were altered in comparison to control and an overall increase in the elastic fibres of the pelvic connective tissues was noted. Alterations in the elastic fibres within the dermis of patients were also identified and suggests that a systemic disorder of connective tissue may account for the development and progression of the condition in some individuals. The pelvic connective tissues themselves were noted to have a number of changes with respect to the disorder, an increase in MMP 1 was noted and this was linked to a reduction in connective tissue supports surrounding the rectum. The growth factor TGF β may play a role in modulating this process and a reduction in the expression of this growth factor was found to be associated with the development of external prolapse. We found that cellularity was increased in response to the process of prolapse in females, but in males this process was attenuated. In addition it was also noted that in some females the cells did not differentiate to the type normally identified in the pelvic connective tissues.

The experimental findings suggest that in some individuals a combination of a systemic connective tissue disorder, altered collagen ratios and an environment favouring tissue degradation may account for the development and progression of rectal prolapse.

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Chapter 1- Introduction

1.0 Overview of disorder

Rectal prolapse is part of a spectrum of pelvic floor disorders characterised by altered rectal anatomy. Two main subtypes are recognised; internal and external rectal prolapse(1). In the latter condition a full thickness external protrusion of the rectum through the anus occurs consisting of all layers of the rectal wall (figure 1). The former condition is less well defined, it consists of internal rectal intussusception whereby the proximal rectum telescopes internally into the distal rectum, but not beyond the dentate line(2). Patients with the former condition will typically present with symptoms of a perineal mass, which may ulcerate and bleed and be associated with disruption of normal continence. Some patients are able to manually self reduce this mass, turning the condition into a chronic problem. Others may not be able to do so, in which case the patient will usually be hospitalised for further management.

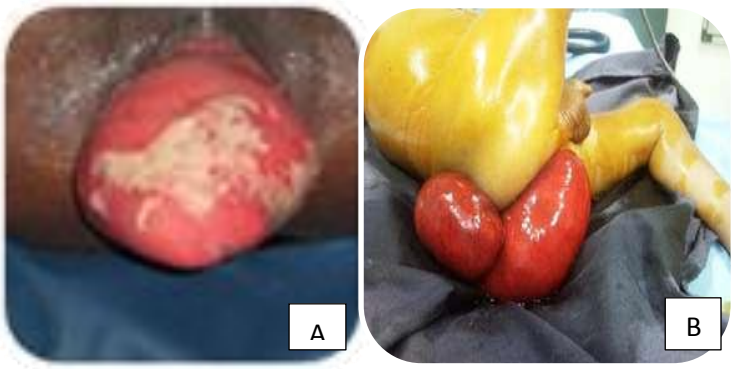


Figure 1 External rectal prolapse

Two clinical images demonstrating the disease spectrum of rectal prolapse. In image A an elderly female presents with an external rectal prolapse, the rectum is seen to lie externally. In image B a child has developed a full thickness external prolapse.

Patients with internal rectal prolapse may present with a number of symptoms including, chronic pelvic pain, abdominal bloating, obstructed defecation, chronic constipation and faecal incontinence(3-5). The diagnosis of internal rectal prolapse is usually made by undertaking a

number of diagnostic investigations including defecating proctography (figure 29) and sometimes examination under anaesthesia using a circular anal dilating device (CAD)(figure 12). Most cases of internal rectal prolapse can be diagnosed by a combination of these two diagnostic modalities. Because of the tendency for internal rectal prolapse to present with vague and sometimes, non specific symptoms, patients may suffer symptoms for many years(6).

Rectal prolapse is rarely an isolated condition and women may also suffer from other prolapse related pelvic floor disorders, including uterine prolapse (figure 2) and stress urinary incontinence(1, 7-9). In the former condition both internal and external uterine prolapse may occur. Stress urinary incontinence manifests itself as a failure of the inability to retain urine within the bladder during periods of raised intra abdominal pressure such as may occur during coughing or sneezing. In this case involuntary leakage of urine may occur(10).

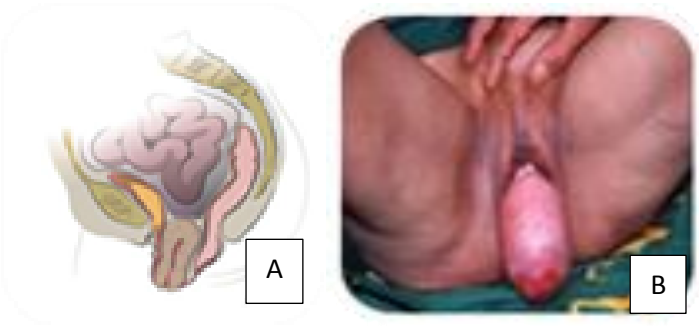


Figure 2- Uterine prolapse

These images demonstrate uterine prolapse. In image A the schematic demonstrates the process of total pelvic floor failure with both rectal and uterine prolapse. In image B the patient has developed an external uterine prolapse.

Patients suffering from these disorders may be referred to three main areas of surgical practice, urology, gynaecology or coloproctology depending upon the symptoms and organ system affected.

1.1 Anatomy of the pelvic floor

The abdominal cavity is a best considered as a large octagonal shaped structure. Superiorly the abdominal contents are constrained by the diaphragm with foramina transmitting the oesophagus, aorta and inferior vena cava. Posteriorly the spinal vertebrae and associated muscles form a robust layer. Anteriorly the abdominal wall muscles running from the rib cage to the pelvic bones form a dynamic three layered support. Defects in this anterior wall are limited to the deep and superficial inguinal rings which transmit the testicular cord structures in males and the uterine round ligaments in females. Inferiorly the pelvis is contained within the pelvic bones, the shape of which varies according to sex, females typically have a gynaecoid pelvis (figure 3) which is wider and shallower, thereby facilitating parturition. In males the pelvis is more android in shape, typically deeper and narrower. In both sexes the pelvic floor is composed of the levator ani muscles which contain foramina to transmit the rectum and vagina(11).

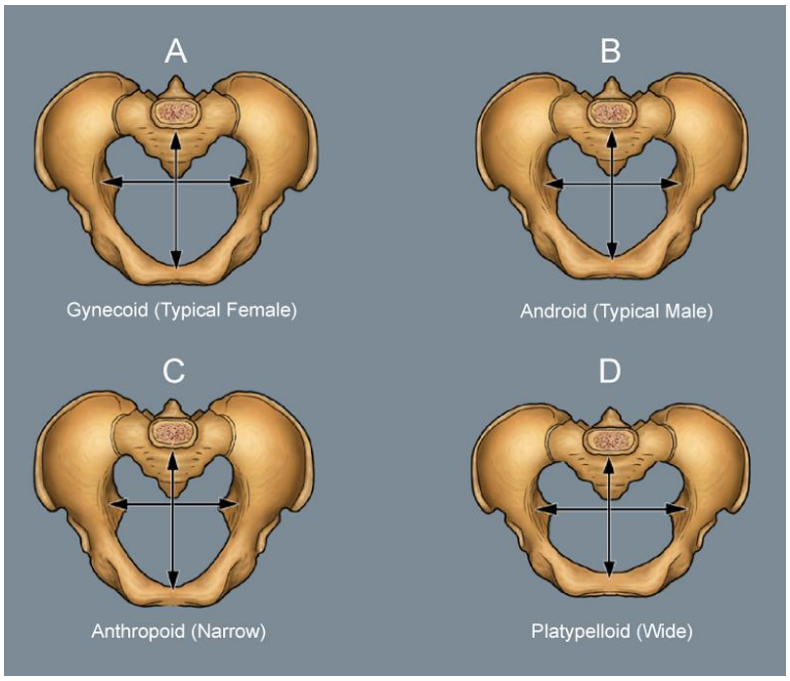


Figure 3 Pelvic morphology

The female pelvis (A) is wider and shallower than the male pelvis (B).The wide pelvis (D) is often seen in rectal prolapse. (From www.anatomytv.com)

The abdominal viscera may either lie within the peritoneal cavity (intra peritoneal) or outside the peritoneal cavity (extra peritoneal). The rectum and uterus have components that are both intra and extra peritoneal, the bladder is extraperitoneal. The sigmoid colon is continuous with the rectum and is an intra peritoneal structure that has a mesentery. The point of transition between

the sigmoid colon and the rectum is termed the rectosigmoid junction and may be identified by the absence of teniae coli on the bowel surface(11).

Humans are bipedal mammals and therefore will typically be orientated in such a way that the pelvis is the most inferior structure and gravity will tend to allow the mobile abdominal contents (mainly small bowel) to rest within it. This latter point also illustrates the requirement that the rectum and uterus share in needing support to prevent them from falling through their respective foramina. It is this latter point which lies at the heart of prolapse disorders. The other abdominal foramina may also be the site of herniation, for example the inguinal canal (inguinal hernias) and the oesophagus (hiatus hernia).

1.2 Mechanisms of pelvic organ support

1.2.1 Rectum

At the point of the rectosigmoid junction the proximal rectum is still a relatively mobile intra peritoneal structure. Support is deficient anteriorly and posteriorly is linked by a mesentery to the posterior abdominal wall(12). At the level of the peritoneal reflection the rectum becomes an extra peritoneal structure and is then surrounded by fatty tissue which forms the interface between the rectum and its respective supporting structures(13). The outer layer of this fat filled mesorectum is continuous with the endopelvic fascia, which overlies the muscles of the pelvic floor. Postero-inferiorly this layer is well defined and is termed Waldeyers fascia (figure 4). It is present in both sexes.

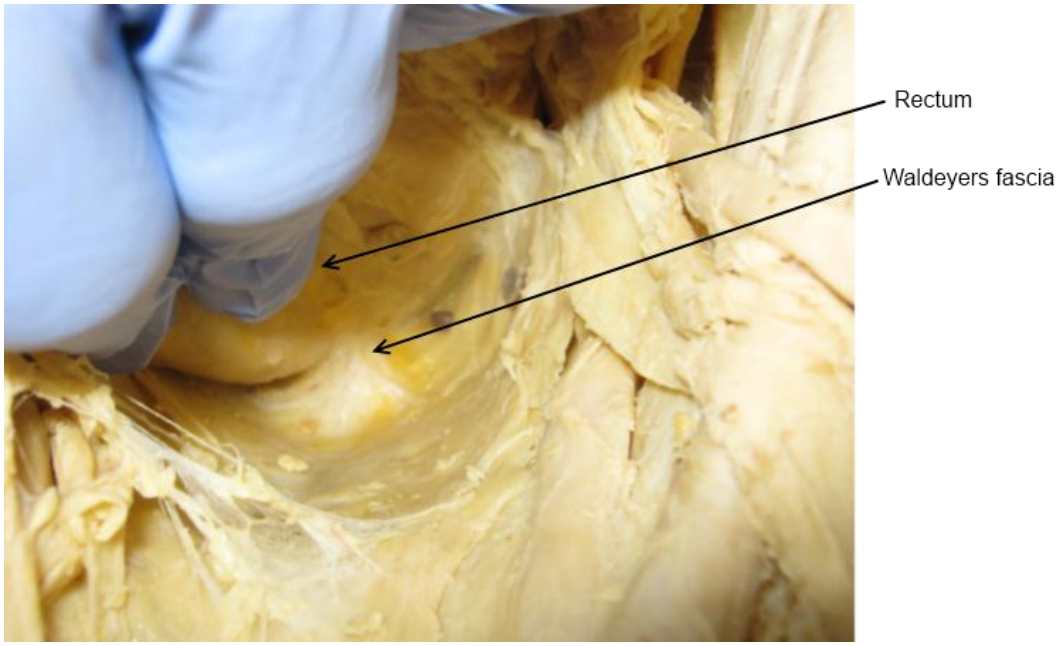


Figure 4 Posterior fascial supports of the rectum

In this dissection the rectum is viewed from the level of the sacral promontory. The proximal posterior rectum has been mobilised. Laying inferiorly and illustrated by the arrow is the thick condensation of Waldeyers fascia.

Anteriorly the endopelvic fascia also condenses to form an anatomical barrier between the rectum and prostate gland in males (Denonvilliers fascia)(11). In females this layer is usually termed the recto vaginal septum (figure 23)(14). The endopelvic fascia has been shown to overlie almost all of the pelvic sidewall structures(15, 16). The work that has been undertaken in relation to the structures surrounding the rectum has largely been driven by a desire to improve the outcomes of surgical resections of rectal cancer. For this reason, most studies have not taken an interest in the structural composition of these connective tissue layers, or how they interrelate with other pelvic connective tissues. It is unclear how, or whether, these pelvic connective tissues have any connection with the rectum at the level of the peritoneal reflection.

1.2.2 Uterus

The mechanisms of uterine support are far better understood than those of the rectum and far more clearly delineated(17). The uterine body is suspended by the broad ligaments, although they are chiefly composed of peritoneum and endopelvic fascia rather than the tough ligaments more commonly encountered in the muscular extremity. Inferiorly, at the level of the cervix, the

supporting structures are more robust and comprise; the round ligament, utero sacral ligaments and transverse cervical ligaments(18). These structures are thick and easily palpable on internal examination(13). The utero-sacral ligaments are the most robust and pass posteriorly to attach to the sacrum, although more detailed anatomical studies have shown a degree of variability with regards to their insertion sites (19).

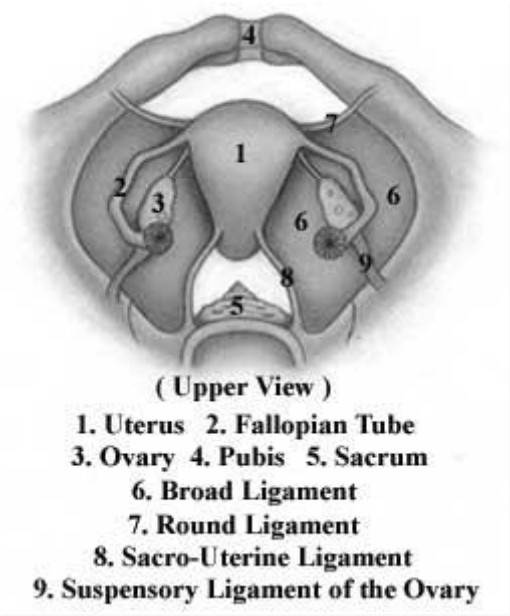


Figure 5 Topography of female pelvic floor anatomy

The uterus is supported by a number of ligaments which may fail causing prolapse.

Image from www.mum.org/fennel.

The importance of these ligamentous structures is highlighted by Delancey(17). Posterior to the vagina the rectovaginal septum (c.f. Denonvilliers fascia)(figure 21) serves as an anatomical boundary to the rectum. Anteriorly the vagina is supported directly by the endopelvic fascia.

1.2.3 Urethra

The urethra is located anteriorly and is a wholly extraperitoneal structure. In females it is extremely short and supported directly by the endopelvic fascia. The location of the bladder neck is important as when it is correctly located any increase in intra abdominal pressure will be transmitted to the bladder and urethra jointly. When displaced, the urethral pressure will not equate to bladder pressure and stress incontinence may ensue(10). In males, the longer urethra, coupled with better developed sphincter mechanisms provide a far better barrier to the involuntary passage of urine.

1.3 Disease spectrum of rectal prolapse

1.3.1 External prolapse

External rectal prolapse is the most obvious variant of the condition. Irrespective of the mechanism, the support for the rectum has failed and the rectum protrudes externally (figure 1). It may frequently protrude following minor increases in intra abdominal pressure. The length of the protrusion may fluctuate, and will typically increase with time(20). Symptoms are variable, though most patients will complain of a lump. The "typical" patient with an external rectal prolapse is an elderly, multiparous female(4). A number of potential treatments are available for the condition. These include external procedures, such as Delormes operation, in which the redundant rectal mucosa is excised and the underlying tissues are plicated. This operation is relatively safe, however, recurrence rates of over 30% at 5 years are quoted(21). For the "typical" rectal prolapse patient this may be an acceptable compromise, since approximately 70% will not recur and some patients may die of unrelated causes in the interim. However, greater patient awareness, and greater longevity has resulted in patients presenting earlier and living longer following surgery for external rectal prolapse. For these patients a different procedure, a rectopexy, may be more appropriate. In this procedure the rectum and its supports are repaired from within the abdomen and the surgical repair reinforced by the placement of a prosthetic mesh(22).

1.3.2 Internal rectal prolapse

Included under this term are rectal intussceception, enteroceles and rectoceles. These disorders may occur as a single entity or as part of a constellation of defects. The concept of intussceception has already been described in section 1.1. An enterocele is a defect which occurs when the small bowel presses onto the rectal wall, which then intusscecepts with a loop of small bowel lying outside the rectum but within the intusscicipiated segment. Enterocele is reported to be a marker of severe pelvic floor weakness and are usually associated with other pelvic floor events(23). They are also present in up to 46% patients with external rectal prolapse(23).

Enteroceles may occur in patients of both sexes and irrespective of parity. They are, however, most common in those women who have undergone hysterectomy.

Rectoceles are a disorder exclusive to women. In this condition the rectovaginal septum is the site of the defect and the anterior aspect of the rectum is then able to herniate through into the vagina. This will, in turn, result in pelvic outlet obstruction and symptoms of obstructed defecation(24). Where they are an isolated event they may be treated with local repair or the placement of a pessary. The extent to which rectoceles progress to rectal prolapse is not well described.

1.4 Pathogenesis of rectal prolapse

The development of rectal prolapse is a complex process and includes a many factors. Some are linked to initiation of the process, such as parity and others are more likely to be involved in disease progression. Aside from parity the processes involved in tissue turnover and repair will be considered.

1.4.1 Effect of parity on the development of rectal prolapse

So far the structures supporting the normal rectum have been considered. In order for rectal prolapse (or any other pelvic organ prolapse) to occur these structures must fail. A causal relationship between parity and pelvic floor disorders has long been appreciated. The nature of childbirth is an inherently destructive process. The delivery itself may be complicated by tears to the pelvic muscles and perineum, be they from the foetus or iatrogenic. Difficult and complicated labours may require the use of instrumental delivery, together with episiotomy. Although such defects are usually repaired at the time of delivery, these repairs are usually only undertaken with respect to external structures, such as the skin, and anal sphincter (if involved). As previously described, many of the structures supporting the uterus and rectum are more proximally located. No attention is paid to repairing these following delivery, nor would it be practicable or safe to do so. It is thus not surprising that vaginal delivery has been associated with an increased risk of surgery for pelvic organ prolapse compared with caesarean delivery (2.2% Vs 0.2%)(25).

Studies documenting the changes in the pelvic connective tissues following childbirth or injury are sparse. One cohort study evaluating changes in the prolapsed uterus following delivery did not find any evidence of increased risk of external uterine prolapse after vaginal delivery, and instead proposed that the main changes to pelvic connective tissues occur following the menopause(26). However, this group do acknowledge that internal uterine prolapse to the hymen is more common following vaginal delivery. This would link with the disease mechanism proposed by Delancey in which he likens the situation to one of a ship in a dry dock supported only by side ropes(17). Taken overall the literature is in agreement that vaginal delivery increases the risk of prolapse. However, the precise mechanisms and exact changes remain unclear. The effects of parturition in relation to the supports of the rectum are not well understood, although as with uterine prolapse an increased risk of developing the condition in multiparous women is noted(25). Whilst the relationship between vaginal delivery and pelvic organ prolapse is clear, other mechanisms must account for the occurrence of the condition in males, nulliparous women and children.

1.4.2 Effect of age on the development of rectal prolapse

The prevalence and incidence of rectal prolapse increases with age, our own data suggest that the peak age for the development of the condition is in the 5th decade. Whilst the condition may occur in children and young adults these are relatively rare and should prompt the clinician to search for an underlying connective tissue defect as described in section 1.9.

1.4.3 Structural failure of the pelvic tissues

The development of rectal prolapse occurs because the connective tissue structures described in section 1.2 have failed. Whilst parity is one mechanism by which these structures may fail there are other factors which could also be responsible. Certain individuals, for example, those suffering from a connective disorder are more prone to developing pelvic organ prolapse. This risk is increased even in those for whom parity is not a relevant predisposing factor. Uterine prolapse has been associated with changes to several of these structural supports (as described

above) and it is plausible that some of these aetiological factors are shared in the development of rectal prolapse. The structural supports that fail and give rise to pelvic floor failure are composed of a number of components of the extra cellular matrix and thus these will be considered further.

1.5 Extracellular matrix

The extracellular matrix (ECM) is composed of a large set of diverse macromolecules, which differ in structure and function(27). The composition and organisation of the ECM is tissue specific. The ECM is synthesized and turned over by the constituent cells of each tissue. The cells thus both produce the macromolecular components and also proteases able to degrade all macromolecules of the ECM. In healthy tissues, rates of synthesis and degradation are in balance, but in pathology degradation overtakes synthesis (28, 29)

The main ECM components studied in pelvic floor disorders are collagen, elastin, and proteoglycans(27). The tissues also contain fat. Water content varies depending upon ECM structure and composition. The balance of these factors depends upon the tissue site and its function. For rectal prolapse to occur the extracellular matrix has, by definition, failed at a mechanical level. The components of the ECM will now be considered in relation to the condition.

1.5.1 Collagen

Collagen is the most abundant protein in the body(30).Up to 28 types are currently recognised, each being a separate gene product with the common feature being a formation of a triple helix with at least one region possessing where the amino acid glycine at every third position (gly-x-y)(30). This configuration allows collagen to form a tight helical structure due to the low molecular weight of the glycine residue.

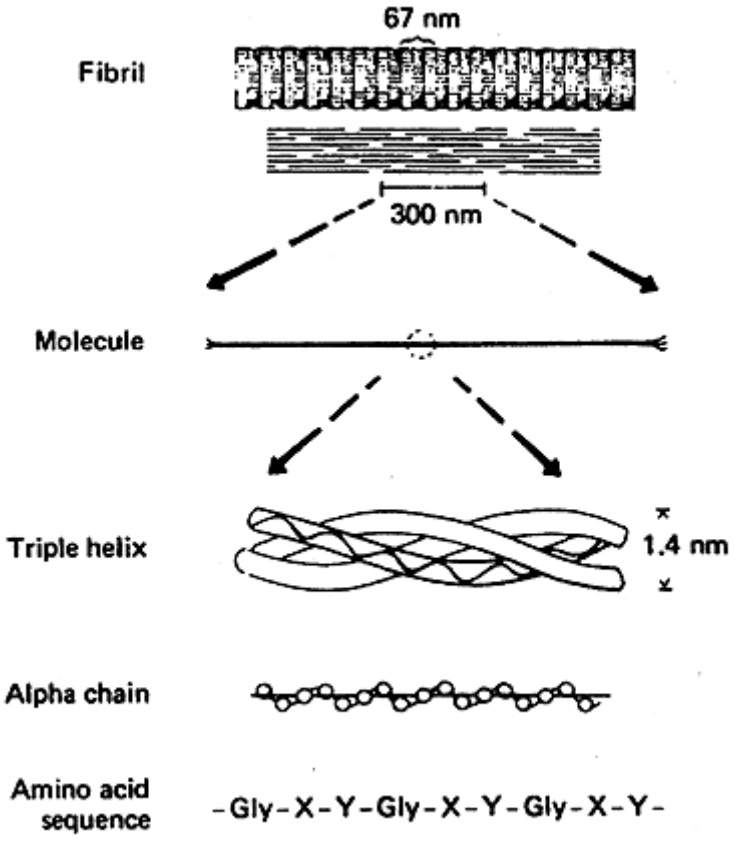


Figure 6- Collagen structure

The Gly-x-y configuration of collagen is able to result in formation of an alpha chain and then a helix.

Image from www.chemistrygravitywaves.com

The other two amino acids (x and Y) most frequently encountered are proline and hydroxyproline. Intracellularly the hydroxyproline chains form disulphide bridges that provide structural stability and result in the formation of a right handed helix. Once synthesised it is released into the extracellular matrix by the golgi body. Once outside the cell the collagen units are assembled into fibres (figure 4). In fibrillar collagen these tropocollagen units are assembled end to end, this progresses from one tropocollagen unit to the next is staggered and this staggering is visible by light microscopy as bands. Cross links, which stablise the fibrils, are formed between adjacent fibrillar units(30). Further cross-links formed during ageing by non-enzymatic glycation of collagen 1, may influence the mechanical properties of collagen adversely (31)

Of the 28 collagen subtypes; the fibrillar collagens type 1 and 3 are most prevalent in the pelvic supporting tissues(31-33) and in many other tissues from bone to skin. In almost all cases a combination of these two subtypes are present (34). Type III is now thought to be co-distributed with type I collagen and may act to regulate fibril size(35)

Two mechanisms exist for the maturation of collagen. The first involves the enzymatically controlled lysine aldehyde cross links(36). The initial enzymatic controlled divalent cross links dehydro-hydroxy lysinorleucine and hydrolysinoketo-norleucine are converted to trivalent cross links, histidine-hydroxy lysinonorleucine and hydroxylysinyl-pyridonline as maturation proceeds. This maturation of cross links enables the maturity of a given tissue to be assessed and formed the basis of Jackson's studies and observations that the collagen of pelvic connective tissues in uterine prolapse was immature(31).

Mature collagen is susceptible to non enzymic cross linking, the Maillard reaction(36, 37). This occurs via the random addition of glucose molecules to the collagen cross links. The glycation products thus formed may then form further intermolecular cross links. This results in the accumulation of advanced glycation end products (which accumulate with age). This altered collagen may have altered biomechanical properties, which in the setting or pelvic organ prolapse disorders may contribute to the disease(38).

1.5.2 Collagen in pelvic organ prolapse

A number of studies have evaluated the potential role of collagen in the development of pelvic organ prolapse. One of the main complexities in comparing the impact of collagen in pelvic organ prolapse is that all studies vary considerably in relation to sampling sites, assay methods and study population. It is therefore unsurprising that some of the results are somewhat conflicting. An overview of the changes seen in collagen in patients with pelvic floor prolapse is given in Table 1. Of note, there is no data relating to the changes in collagen in rectal prolapse disorders.

Table 1 Studies focussed on collagen changes in pelvic organ prolapse disorders

Collagen types	Patient population	Tissue studied	Findings	Reference
1 and 3	Nulliparous women(n=36) with GSI Vs. Control (n=25)	Paraurethral connective tissue	Decrease in type 1: type 3 Reduced cross links	(33)
All (NOS)	Premenopausal with uterine prolapse (n=8) Vs Control (n=10)	Vaginal epithelial tissue	Decreased total collagen Increased glycation Increased cross links	(31)
1	Uterine prolapse (n=23) Vs. Control (n=21)	Uterine parametrium	No difference in type 1 collagen content. Changes in morphology of collagen	(39)
1 and 3	Grade 3 / 4 haemorroids (n=31) Vs. Control (n=20)	Haemorroidal tissue and skin from same area in control	Decreased type 1: type 3 ratio in disease state.	(40)
All (NOS)	Uterine prolapse (n=24) Vs. Control (n=21)	Cardinal and uterosacral ligaments	Increased total collagen	(41)
All (NOS)	Uterine prolapse (n=14) Vs. Control (n=17)	Cervical ligaments	Decreased total collagen	(42)
1 and 3	Uterine prolapse (n=25) Vs. Control (n=16)	Uterosacral ligaments	Increased type 3 in prolapse. No change in type 1.	(43)
1 and 3	Premenopausal with uterine prolapse (n=16) Vs. Control (n=15) Post menopausal uterine prolapse and HRT (n=23) Vs. Post menopausal with uterine prolapse and no HRT (n=23)	Vaginal tissue	Increased total collagen in pre menopausal with prolapse and post menopausal without HRT. Decreased total collagen in uterine prolapse with SUI. Increased type 3 collagen in uterine prolapse. No change in type 1 collagen.	(44)

Collagen types specified where specific types were the main focus of the work, the term NOS refers to studies in which total collagen type or morphology was evaluated with no attempt to identify specific types.

Of these studies, those by Jackson *et al* .for instance studied tissue turnover in urinary stress incontinence. They found total collagen content (per gram of tissue) was considerably reduced compared to controls but that immature cross-link density increased, indicating collagen

biosynthesis was up regulated but could not compete with rate of degradation the actual turnover and degradation of collagen that was present. It is unclear whether the changes represent changes in collagen alone or a relative increase in other components of the ECM (such as fat). When elastic fibre content (using desmosine assays) were studied in this group, no differences were found. In a different study Barbeiro *et al.* focussed on type 1 collagen changes using immunohistochemistry performed of tissue harvested during hysterectomy for prolapse. Here, they found no difference in type 1 collagen content. But they did identify changes in collagen fibre morphology in the patients suffering from prolapse, with a tendency towards more fibre disruption being present in those suffering from the condition.(39)

Collagen content is not only determined by the rate of collagen biosynthesis but also by the rate of collagen degradation by metalloproteinases. This area will be discussed below in section

1.6. The data considered in this section is taken from individuals with a given disease process. It is difficult on the basis of this data alone to determine whether the changes identified in nulliparous women with pelvic floor disorders are part of a local phenomenon or result from an inherent connective tissue disorder.

1. 5.3 Elastic fibres

Elastic fibres are complex tissue structures consisting of amorphous elastin core and surrounded by a mantle of fibrillin rich microfibrils(45). They confer upon tissues the properties of elasticity and resilience(46). Elastic fibres are laid down during foetal development and are designed to last a lifetime. They may be deposited in tissues, for example during the latter stages of human wound healing(47). They may also be degraded by tissue enzymes. This latter process may contribute to the development of mechanical failure of certain tissues and has been implicated in the development of conditions such as aortic aneurysms(48). Interface molecules , such as fibulin may serve a role as linkage between the microfibrils and elastic core. The presence of fibulin 5 seems to be required for normal elastic fibres synthesis; in an elegant and detailed study,

Budatha *et al*. studied fibulin 5 knockout mice and found that the knockout mouse has abnormal elastic fibre organisation and excessive levels of MMP 9 (49) These fibulin 5 knockout mice had a high risk of the development of uterine and rectal prolapse(50).

The stability of elastic fibres is governed by the activity of lysyl oxidase 1 like protein (51).In LOX 1 knockout mice there is a greatly increased incidence of uterine prolapse (52).

Changes in elastic fibres of patients with uterine prolapse and stress urinary incontinence are summarised in table 2.

To date, no studies have been undertaken evaluating the form and distribution of elastic fibres in rectal prolapse, although, they have been identified in increased quantities and altered deposition patterns in the sub mucosal layer of the rectum in rectal prolapse patients (53). Whether they are the cause or the result of the prolapse process is difficult to determine, although their immature morphology would point to the latter.

Table 2 Summary of studies evaluating elastic fibres in pelvic floor disorders.

Population studied	Tissue analysed	Findings	Author
Post menopausal women with uterine prolapse (n=4) Vs. Control (n=4)	Fibroblasts from cardinal ligaments	Decreased mRNA and tropoelastin production	(54)
Women with uterine prolapse (n=33) Vs. Control (n=25)	Cardinal ligaments	Decreased elastin content	(55)
Women with SUI or prolapse (n=12) Vs. Control (n=15)	Periurethral vaginal wall	Decreased elastase inhibitors and decreased elastin content.	(56)
Women with uterine prolapse (n=29) Vs. Control (n=30)	Uterosacral ligaments	Decreased elastin content	(57)
Premenopausal women with uterine prolapse (n=8) Vs. Control (n=10)	Vaginal epithelium	No change	(31)

Women with uterine prolapse (n=23) Vs. Control (n=15).	Vaginal wall	No change	(58)
Women with uterine prolapse (n=33) Vs. Control (n=10)	Vaginal wall	Decreased elastin content	(59)
Women with uterine prolapse (n=24) Vs. Control (n=21).	Uterosacral and round ligaments	No change	(41)

The consensus from these studies appears to be that elastin is decreased in uterine prolapse disorders. However there have been no studies of elastic fibre morphology, biosynthesis or turnover at a distant non disease site to determine whether the changes towards a low concentration of elastin seen in prolapsed tissues result from an inherent disorder, or if elastin falls as a consequence of environmental changes arising from the prolapse. Changes in mechanical stress for instance, could alter elastin turnover; elastin production by periodontal ligament cells subjected to shearing forces in culture was lower than in control cells (60). In the vagina increased tropoelastin is seen following partuition (61, 62). The induction of elastin gene expression in prolapse patients is absent or reduced in the setting of uterine prolapse demonstrating a heterogenous response of the pelvic tissues to the events of prolapse(54) This may, however, not be applicable to the situation in pelvic connective tissues in rectal prolapse as increased elastic fibre production is a consistent feature within the colonic mucosa (53).

1.6 Turnover of the extracellular matrix

Turnover of the ECM in the normal individual is a coupled process of matrix degradation and synthesis. In the healthy situation these processes will be balanced such that there is no net tissue loss and tissue architecture is preserved. In diseases characterised by degradation of the ECM this process becomes uncoupled such that matrix degradation exceeds matrix synthesis. The turnover of the extracellular matrix is primarily the result of action of the matrix degrading enzymes, matrix metalloproteinases (MMP's). This multigene family of over 23 secreted and cell surface zinc-dependent endopeptidases process or degrade numerous substrates at neutral

pH(63). MMP's are secreted molecules with several conserved domains. They all share a catalytic domain, which is shielded off in the inactive form of the enzyme by the prodomain. This propeptide interacts with the catalytic region through a conserved cysteine residue. Located within the catalytic domain, and common to all MMP's is a Zn^{2+} ion. One mechanism of activation involves exposure of this catalytic region by cysteine displacement(64). The catalytic activity most probably occurs via a common mechanism in which a zinc bound water molecule is activated, by the carboxylate group of the conserved glutamate residue. The activated hydroxyl group can then attack a polarised carbonyl group on the substrates scissile bond(65).

MMP's may be classified into the following main groups:

- Collagenases
- Gelatinases
- Stromelysins
- Matrilysins

These groups provide some insight as to the substrate specificity. The structure of MMP's is intrinsically linked to their biological function. With the exception of MMP 7 all MMP's contain a C- terminal haemopexin like domain that functions as a recognition sequence for the substrate. The haemopexin domain has a structure similar to a four bladed propeller, with each blade consisting of 4 antiparallel β sheets and an α helix. This is an absolute requirement for the degradation of triple helical collagens.

In this thesis attention will focus on MMP 1, 3,7 and 9. Of these MMP 1 has been extensively investigated with respect to connective tissue changes in pelvic floor disorders (table 3). The other MMP's have been less frequently studied in relation to pelvic floor disorders. However, MMP's 7 and 9 have both been implicated in the development and progression of other disorders affecting the ECM(66-68), but have not been studied in relation to pelvic floor disorders.

1.6.1 Matrix metalloproteinase 1

The first MMP to be discovered, this enzyme is considered a true collagenase and is thus capable of cleaving fibrillar collagens. Like all MMP's it is secreted in an inactive form. Activation primarily occurs via proteolytic cleavage of the inhibitor by mast cell tryptase, urokinase or by other matrix enzymes, such as MMP 3(69). The factors controlling MMP release have not been well characterised in the setting of pelvic floor disorders. In conditions such as malignancy, growth factor activation of MMP activity is well described (70). Integrins present on the cell surface will facilitate identification of matrix components and help to localise MMP release to the site of action (70).

The ECM in patients with uterine prolapse (and therefore possibly rectal prolapse) is actively remodelling and therefore activity of MMP 1 in these tissues would seem likely. Several researchers have studied this enzyme in patient s with uterine prolapse and stress urinary incontinence (see table 3).

Table 3- Summary of studies evaluating MMP's in pelvic floor disorders

Population and sample size	Tissue analysed	Findings	Authors
Women with prolapse and/ or SUI (n=7) Vs. Control (n=15).	Vaginal epithelium	Increased MMP 1 mRNA	(71)
Women with prolapse (n=17) Vs. Control (n=18).	Uterosacral ligaments	No change in MMP1 expression	(43)
Women with prolapse (n=20) Vs. Control	Uterosacral ligaments and vaginal epithelium	Increased MMP1 expression in both tissues	(72)
Women with prolapse (n=46) Vs. Control (n=49)	Uterosacral ligaments	Increased MMP 1	(73)

All the above studies studied the presence or absence of MMP1 in tissues using immunohistochemistry, western blots or its mRNA expression. They did not attempt to correlate

MMP 1 with stage of disease or between its active and latent forms. It is therefore not possible to state whether it was exerting a biological effect.

1.6.2 Matrix metalloproteinase 3

MMP 3 differs from MMP 1 in that it is not a pure collagenase. Formally viewed as a stromelysin MMP it lacks the haemopexin domain, present in MMP1 and for that reason cannot degrade fibrillar collagen (74). It has a broad substrate specificity including; fibronectin, laminin, elastin, collagen IV and proteoglycans (75). Its release may be triggered by cytokines such as TNF α. It's ability to degrade basement membrane may play an important role in modulating inflammatory activity. In addition it is also able to activate MMP 1. To date no studies have been undertaken studying the role of MMP 3 in pelvic floor disorders. Most research has concentrated on its role in neuroinflammatory disorders and neurodegenerative diseases (74).

1.6.3 Matrix Metalloproteinase 7

MMP 7 is a smaller molecule than other MMP's because it lacks the C-terminal haemopexin domain. It is secreted as a 28kDa pro-enzyme that is activated within the extra cellular matrix, for example, by other MMP's (76). It has a similar series of substrates to MMP 3 including; elastin, fibronectin, type IV collagen, vitronectin and aggrecan (77). Because it lacks the haemopexin domain it is unable to cleave fibrillar collagen. It has a number of other important roles, in the intestine it serves an immunological role activating the defence protein, a defensin. It is also capable of activating MMP 9 (78).

1.6.4 Matrix Metalloproteinase 9

MMP 9 is involved in the degradation of components of the ECM and in particular damaged collagen and elastic fibres(71). The overlap between MMP 9 activity and other matrix enzymes was demonstrated in a study of fibulin 5 knockout mice(49) (which have a high rate of pelvic organ prolapse(79)). Those mice receiving the inhibitor to MMP 9 demonstrated elastic fibres of

normal morphology in the pelvic connective tissues suggesting that MMP 9 may be of importance of elastic fibre degradation in prolapse.

1.6.5 Tissue inhibitor of metalloproteinases

Tissue inhibitors of matrix metalloproteinases (TIMP) are endogenous inhibitors of MMP's. The human genome has 4 paragolous genes encoding for 4 different TIMPs , these vary in their MMP specificity, although there is considerable overlap in biological effect between them. In addition to inhibition of MMP's, the TIMP family also exert direct biological effects in their own right, including inhibition of angiogenesis and stimulation of cell proliferation(80). The inhibition of MMP's by TIMP's occurs via the insertion of a highly conserved molecular ridge into the active site of the MMP enzyme(80). This conservation of structure accounts, in part, for the overlapping biological activity. The decrease of TIMP's has been identified in individuals with single compartment pelvic floor failure (isolated uterine prolapse)(81). To date, TIMP activity has not been studied in the pelvic connective tissues of rectal prolapse patients. It would, however, seem plausible that decreased TIMP activity is likely as this disorder consisting of mechanical tissue failure.

1.7 Growth factor activity in the extracellular matrix

Growth factors comprise a range of substances that serve a crucial role in the control of activities within the ECM. They control many different aspects of cell and matrix, from cell division through to angiogenesis. In the context of pelvic floor disorders the following growth factors will be considered; transforming growth factor β and fibroblast growth factor. Oestrogen, a female reproductive hormone, will also be discussed.

1.7.1 Transforming growth factor β

Transforming growth factor β (TGF β) is a growth factor with a wide range of effects. It is a key mediator in inflammatory and degenerative disorders with effects on many different cell types.

TGF β has three recognised isoforms containing between 390 and 412 amino acids. On secretion from the cell they have a latency associated peptide which is cleaved upon release to confer biological activity. Latent TGF β main remains dormant and inactive in tissues for a variable period of time prior to activation (82). The MMP's may cleave the latent protein from TGF β and thus render it biologically active, both MMP 2 and 9 are capable of doing this (83). Although TGF β may also be activated by low pH and reactive oxygen species it is unlikely that these events would account for its activation in pelvic organ prolapse.

One of the earliest recognised functions of TGF β was its ability to induce cellular apoptosis. Knockout mice for TGF β have overwhelming immune system activation and death (84). In the ECM TGFβ is known to induce differentiation of fibroblasts to myofibroblast subtypes. It also increases the synthesis of various matrix proteins such as collagen. It is interesting that TGF β does not induce the apoptosis of fibroblasts, but rather induces differentiation. This latter event usually only occurs in those tissues with increased tensile forces(85). Myofibroblasts have also been shown to release TGF β in an autocrine or paracrine manner. This latter process is specifically induced by tensile forces within the ECM and may thus have implications for pelvic floor disorders.

The effects of TGF β on the MMP's in the ECM are more difficult to determine. MMP's may in themselves activate TGF β, and TGF β may both increase or decrease MMP production *in vitro* studies have shown this at an mRNA level (86). The net effect of TGF β in the ECM appears to be net deposition of ECM components, with increases of both MMP's (favouring degradation and turnover) and TIMP's (favouring MMP inactivation) (87). Studies evaluating the role of TGFβ in prolapse disorders are limited. In one study Qi

et al studied the cardinal ligaments from patients with uterine prolapse and found that patients with uterine prolapse had lower levels of TGF β than that seen in the control group. They did not see any differences between the different stages of pelvic organ prolapse (88). To date no studies have been undertaken in relation to TGF β in rectal prolapse disorders.

1.7.2 Fibroblast growth factor (basic)

Fibroblast growth factor (FGF) has important roles in wound healing, tumour angiogenesis and embryonic implantation. It is so named because of its ability to induce fibroblasts to proliferate (89, 90). They are subdivided into acidic and basic subtypes according to their response to mitogen signals. In total there are over 22 isoforms of FGF. In the setting of matrix turnover and tissue regeneration bFGF is the most widely studied isoform. FGF is secreted in a form that is able to bind heparan sulphate. This results in binding within the ECM. As a result it is typically localised to the periphery of blood vessels, reflecting its role as an important mediator of angiogenesis. Its binding to the ECM may also help to more precisely target its activities to sites of need, as the active form will only be released at specific sites of injury, for example.

To date, no studies have been undertaken in relation to any pelvic floor disorders and the effects of basic fibroblast growth factor. However, the hypothesis that the process of prolapse results in injury to the pelvic floor tissues would seem to make this an important growth factor to study.

1.7.3 Oestrogen in pelvic connective tissues

Oestrogen is a female sex hormone, which is one of the steroid hormones. The steroidal nature of its molecular structure facilitates its passage across the cell membrane. Once inside the cell it binds to oestrogen receptors. Three types of oestrogen are recognised,

oestradiol is the predominant isoform that is responsible for the regulation of the menstrual cycle(91). During the menopausal years the less biologically active form of the hormone, oestriol, becomes the most predominant type(91). All oestrogens bind to the intracellularly located oestrogen receptors, the activation of which accounts for the biological activity of oestrogens. The many actions of oestrogens fall beyond the scope of this thesis. However, they are recognised as having a role in the tissue turnover and topical oestrogens have been shown to increase vaginal collagen synthesis(92). In women with uterine prolapse a decrease or alteration in the oestrogen receptors have been identified(93-96). The finding of altered oestrogen receptor expression in female pelvic connective tissues may account for the heterogeneous and sometimes poor treatment results that are recognised when topical or systemic oestrogen therapy is administered for pelvic organ dysfunction(97). The data relating to oestrogen receptor expression indicates that these may be altered in some individuals with prolapse and merits further study.

1.8.1 Normal pelvic floor connective tissues

The normal rectum resides in the pelvis and comprises a mucosal layer of tissue lined by mucous secreting goblet cells (figure 7). This is surrounded by a layer of smooth muscle which is arranged in two layers. Outside this region lies the structural supporting tissues. These comprise fat cells that are arranged around the rectum and form an interface with the endopelvic fasica. Cell types identified in these tissues will include; fibroblasts, adipocytes, endothelial cells and nerve cells. The detailed structure and cellular composition of these tissues form part of the thesis and are described in section 3.1.3.

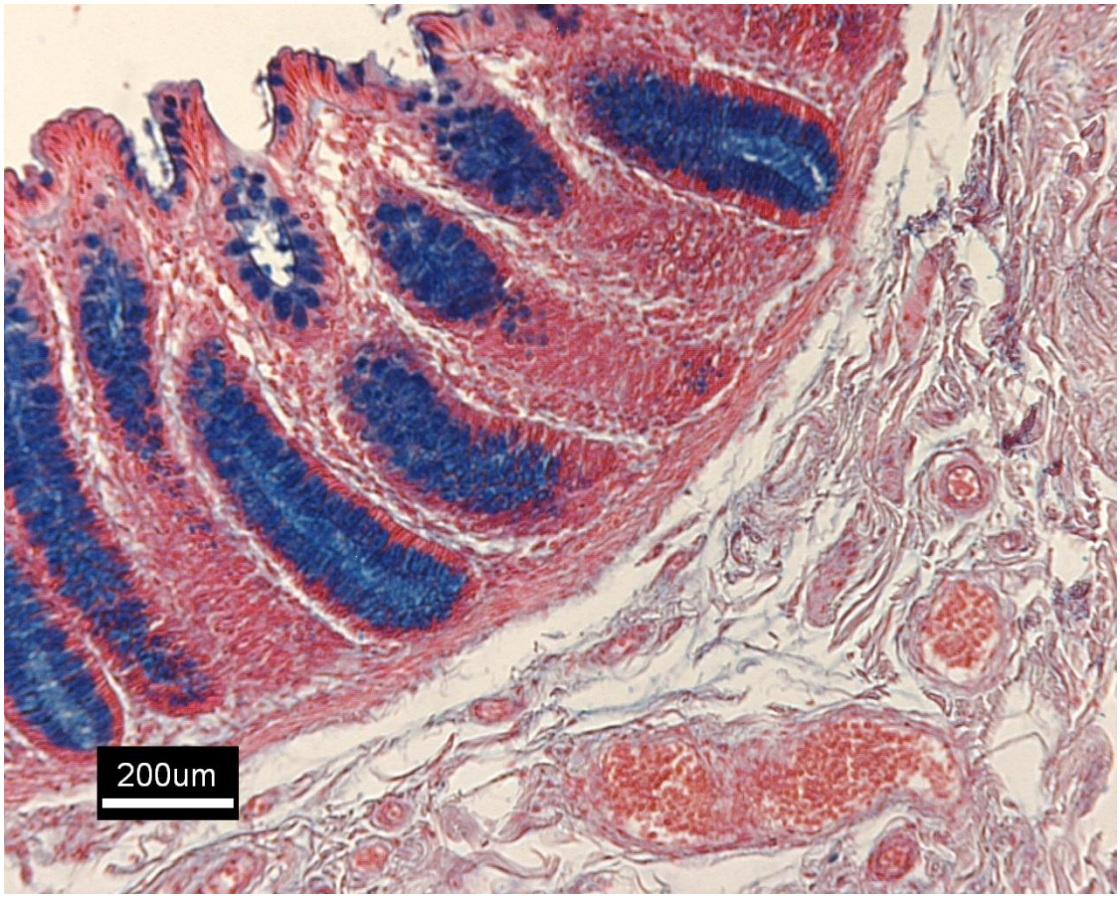


Figure 7- Goblet cells in the rectum

Histological section of the human rectum stained using alcian blue and and nuclear fast red to demonstrate the goblet cells in the rectum. These secrete mucus (stained dark blue).

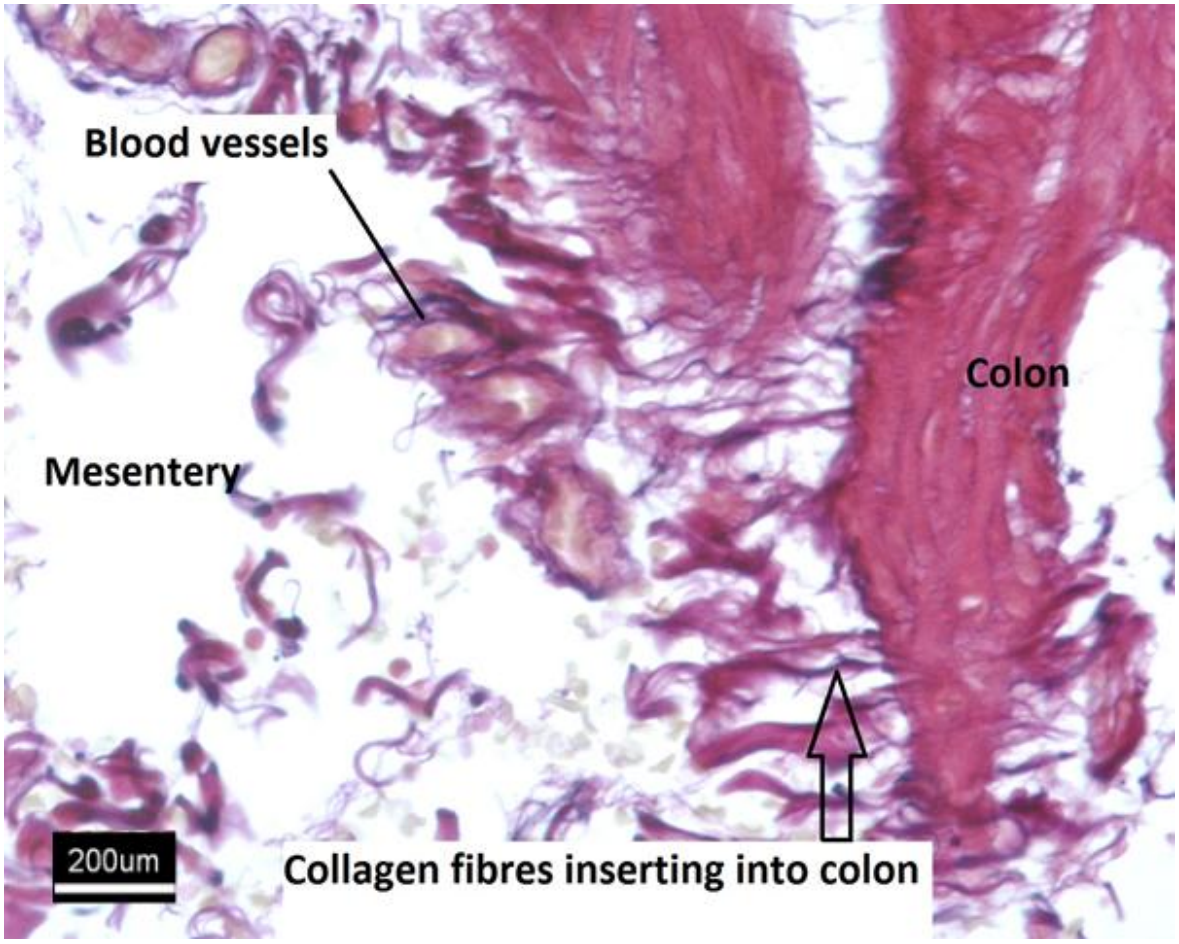


Figure 8- Histological section of colon and supporting tissues.

Transverse section of the distal sigmoid colon stained using elastin van Gieson (collagen = red/ purple, elastic fibres = black). Demonstrating the point of attachment of mesenteric tissue into the mesenteric border of the colon.

1.8.2 Fat in body tissues

The presence of fat in the tissues surrounding the rectum is important in terms of the function of the rectum as a storage organ. Expansion of the rectum is readily accommodated in the surrounding fatty tissues. On a macroscopic scale, marked differences exist in the distribution of body fat in males and females. Males typically have fat distributed in an android pattern with centralised distribution of fat. In females fat is distributed more peripherally (98). The exact internal distribution of this fat is governed by factors such as gestational age at birth, stress (99) and physical exercise (100), with slim fit adult males typically having less visceral fat than other groups. The differences in fat distribution have been linked to cardiovascular disease and the risks of developing type 2 diabetes.

These changes in fat between sexes may be important structurally, since individuals with increased quantities of pelvic fat may have different structural characteristics to those with decreased fat. The relationships between pelvic connective tissue fat and function have not been formally investigated. However, we have often observed that the majority of patients presenting with symptoms of rectal prolapse are generally thin. One potential explanation for this phenomena is the relatively recent finding that adipose tissue itself can be a potent stimulus for tissue remodelling (101). In the context of rectal prolapse the mesorectal fat is likely to play a significant role in the sequestration of growth factors, this will be investigated further in this thesis.

1.8.3 Response of tissues to injury

The process of rectal prolapse can be viewed as being injurious to pelvic connective tissues. In tissues at other bodily sites the process of injury will typically result in a series of stereotyped events, that are designed to organise and either repair or regenerate the injured tissue as efficiently as possible. It is likely that the prolapse process will lead to disruption of the pelvic connective tissues and that this will lead to cellular proliferation and matrix synthesis. No work has been undertaken studying this process in rectal prolapse, so it is not possible to determine what may occur. In uterine prolapse only one study has been conducted evaluating the tissue response to the prolapse itself. In that study it was found that patients with pelvic organ prolapse had significantly lower levels of fibroblasts in their uterosacral ligaments than the control group (41). This would seem unusual since trauma to tissue will typically result in release of growth factors that increase cellularity. However, it may be that the tissues which Kokcu *et al*. studied were contributors to, rather than directly involved in, the prolapse process.

The extracellular matrix of the pelvic floor is composed of connective tissues and populated by a resident cell population. These cells are fibroblasts and are widespread throughout most connective tissues in the body. Normal fibroblasts are spindle shaped cells of mesenchymal origin. In tissues that are subjected to injury or trauma the population of fibroblasts is usually

seen to increase(102). This was not observed in the studies described above, which is unusual. There are two main pathways by which local fibroblast populations may expand. They may either increase as a direct result of proliferation of the resident cell population, or by migration towards chemotactic signals from surrounding tissues and blood vessels. The mechanism by which fibroblast populations either increase or decrease in response to pelvic organ prolapse has not yet been determined.

1.8.4 Myofibroblasts

Myofibroblasts are a differentiated type of fibroblast. They are found in numerous pathological conditions such as fibrotic lung disease, cirrhosis of the liver and malignancy. Physiologically they are present in the normal human colon(103), pelvic connective tissues(104) and in the latter stages of wound healing. Fibroblasts are mesenchymal cells that have a spindle shaped morphology and ubiquitous throughout many tissues in the body(105). They are the source of many ECM constituents, they also secrete a wide range of MMP's and their inhibitors(106). The transition from fibroblast to myofibroblast is complex, these differentiated cells have laid down α smooth muscle actin (106). This confers properties of increased resilience to tensile forces and increased contractility (107).

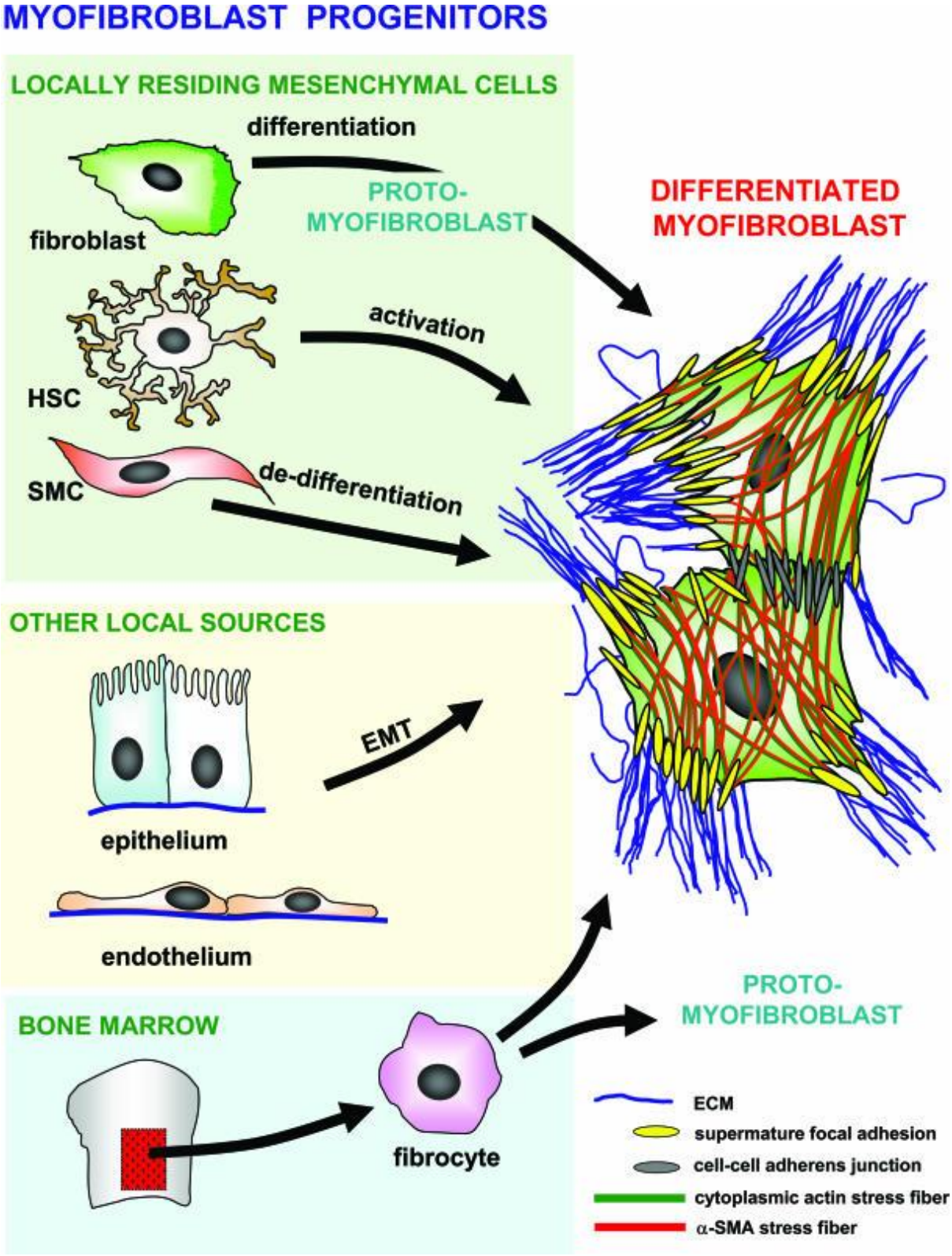


Figure 9 Origin of myofibroblasts (Adapted from Hinz *et al.* 2007 (106))

Key to abbreviations; HSC= stem cell, SMC= smooth muscle cell, EMT= electromechanical transduction.

In order for myofibroblasts to be present at least three conditions(108) need to occur:

1. Accumulation of transforming growth factor β
2. Accumulation of specialised ECM proteins (e.g. spliced fibronectin).
3. Increased tensile forces within the ECM

It is likely that at least some of these criteria are fulfilled by the conditions surrounding cells within the pelvic floor. This is a site that even in the normal situation is subjected to regular and sustained mechanical stress. In many cases previous trauma (e.g. during childbirth) may result in local trauma. Marked and regular changes in force are also present within the human colon and rectum, by virtue of peristalsis. As a result, it is perhaps unsurprising that in the colon myofibroblasts have been shown to be present in the normal situation(103, 109). Whilst myofibroblasts have not been investigated in the setting of rectal prolapse investigations in the *in vitro* setting have been undertaken in relation to uterine prolapse, in this area, the evolution of prolapse is associated with a proportion of myofibroblasts that have an impaired contractile ability (104, 110). These studies would imply that myofibroblasts are part of the normal cellular composition in the pelvic floor connective tissues in women. Determination of function of myofibroblasts *in vivo* would be difficult and therefore for the present time only cell culture results are available. Whilst impaired myofibroblast contractility may be contributory to the propagation of pelvic organ prolapse it is more difficult to envisage that complete loss of myofibroblasts is likely to be a primary causative factor.

1.9 Generalised connective tissue disease and pelvic floor disorders

Several generalised connective tissue disorders are recognised. Those most associated with pelvic organ prolapse include Ehlers Danlos syndrome and Marfans syndrome. These disorders are multisystem disorders affecting the connective tissues. As a result, individuals with these disorders often develop diseases characterised by connective tissue failure at sites of physiological stress, including, aortic aneurysms, cardiac valve diseases and rectal prolapse. These

disorders are of interest in the setting of rectal prolapse research because in some cases the genetic basis of the disorder is understood and may provide a means of better understanding the disorder in the majority of rectal prolapse patients in whom genetic information is lacking.

1.9.1 Ehlers-Danlos syndrome

This consists of a group of inherited defects of connective tissue sharing certain physical features such as hypermobile joints, silky, soft, stretchy and fragile skin which typically heals with paper thin scars. Some types show particular predispositions to short stature, scoliosis and other skeletal abnormalities. There are 8 recognised subtypes. These include types 1 and 2 which represent “classic” Ehlers Danlos in which there are marked skin changes, joint hypermobility, easy bruising and a risk of pelvic organ prolapse. It is inherited in an autosomal dominant fashion. This is a rare disorder with an incidence of 1 in 20,000(111).

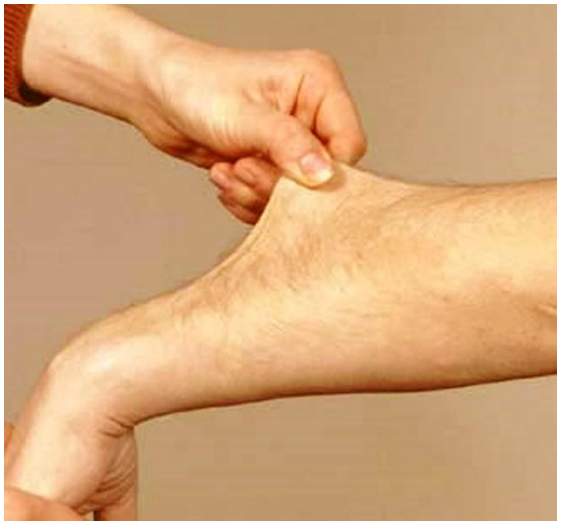


Figure 10 Skin changes in Ehlers Danlos

Ehlers Danlos type 1 and 2 have characteristic skin changes as demonstrated here.

These rarer types share an overlap with EDS type III, otherwise known as benign joint hypermobility syndrome (BJHS)(112). In this variant the key feature is one of hypermobile joints. However, the marked skin changes observed in the “classic “ type EDS are usually absent (111).

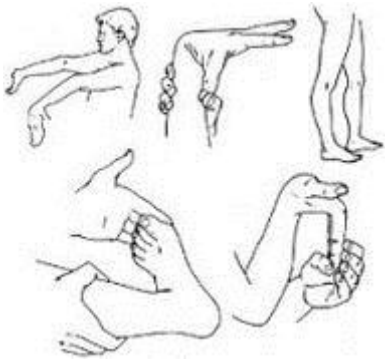


Figure 11 Joint changes in BJHS

BJHS is associated with increased flexibility of joints. Some or all joints may be affected. The degree of flexibility can be objectively assessed using the Beighton scoring system.

In the classic type EDS mutations involving collagen genes have been identified. One group has linked BJHS to changes in type 1 and 3 collagen in pelvic connective tissues in patients with uterine prolapse (113). The importance of BJHS in this study is that a considerable number of patients presenting to clinics with non specific gastrointestinal symptoms may have the disorder (114, 115). Furthermore BJHS has been associated with a number of pelvic floor disorders ranging from chronic constipation through to rectal prolapse (113, 116-120). Given that the true biochemical implications of BJHS are not well evaluated, the diagnosis of BJHS usually relies on a careful history and clinical examination. Amalgamation of features in the history and on examination can be assessed against the Beighton Criteria for diagnosis of BJHS(121, 122).

1.10 Summary

The process of rectal prolapse is complex and almost certainly multifactorial. Almost no research has been undertaken in relation to the pathophysiology of the disorder. Even concepts which have almost become enshrined in surgical theory, such as the effects of pregnancy on the pelvic floor are founded on studies correlating with disease rather than as a result of tissue studies. Research which has been undertaken has been largely clinically based but does suggest a connective tissue aetiology for prolapse disorders(123). Studies undertaken in relation to gynaecological prolapse provide a useful framework for how research into rectal prolapse disorders may be organised and suggest that at the heart of pelvic floor disorders lies a failing in ECM and its various components.

The frequency of these disorders in the general population(21, 124), together with the need for complex surgical interventions, with variable outcomes(125, 126), make this an important topic for further study.

1.11 Aims

The hypothesis underlying the work in this thesis is that connective tissue changes lead to rectal prolapse in both internal and external variants. The overall aim of this work is to determine whether an underlying disorder of the pelvic connective tissues contributes to the development of the condition, its progression and outcomes following surgery. The following specific aims will dictate the nature of the experimental work that will be undertaken.

1. To develop a human model for studying the pelvic connective tissues, so that changes occurring in association with rectal prolapse may be categorised.
2. To outline clinical manifestations that accompany pelvic tissue failure at both a local and systemic level.
3. To develop a method of histologically assessing the pelvic connective tissues and characterising the microscopic changes that occur in relation to rectal prolapse.
4. The pelvic connective tissues will be studied to determine the form and distribution of collagen types 1 and 3. The form and distribution of elastic fibres will also be determined.
5. Enzymes responsible for matrix turnover will be analysed, together with their endogenous inhibitors with respect to location and microscopic effects.
6. Cell signalling methods in relation to modulation of the above processes by TGFβ, bFGF and oestrogen.
7. The impact of the above on the resident cellular population will be determined.

Full ethical approval and informed consent will be obtained from study participants (appendix 1)

Chapter 2- Materials and methods

2.1 1 Assessment of connective tissue status

Patients were clinically assessed to determine whether they fulfilled the diagnostic criteria for benign joint hypermobility syndrome and also to determine their degree of joint laxity. This was performed using the Beighton assessment criteria (121). As part of this assessment it was determined whether they had either normal connective tissue status, joint laxity or benign joint hypermobility syndrome.

Table 4 Beighton scoring system for hypermobility

Criteria	Score
Hands flat on floor with knees straight	1
Bend elbow backwards	2 (1 for each side)
Bend knee backwards	2 (1 for each side)
Thumb back to forearm	2 (1 for each side)
Little finger 90 degrees to angle of hand	2 (1 for each side)

All participants underwent an anterior laparoscopic ventral mesh rectopexy procedure(22). Following the procedure all participants were followed up for a period of 12 months and all additional operative procedures related to their disease were noted. The follow up process was concluded at twelve months because of the timeframe of the project and for resource reasons.

2.1.2 Patient characteristics of benign joint hypermobility participants

For the purposes of data acquisition the patients were placed into two groups. Group 1 were assessed for the effect of benign joint hypermobility on previous surgical interventions for prolapse and general presenting demographics. Patients in the second group constituted those who were able to complete the follow up process during the timeframe of the project. They too were assessed for features of joint hypermobility syndrome, the other sub group consisted of those patients who had rectal prolapse, but no features of joint hypermobility syndrome. Re-interventions were classified as those which required revisional surgery, minor complications such as post operative wound infections and non operative treatments such as biofeedback (a

series of behavioural interventions designed to manage symptoms of pelvic floor dysfunction) were not included.

Patient characteristics

Table 5 - Group 1 Effect of joint hypermobility on previous procedures for prolapse

Group	N	M:F	Median age	Range
Benign joint hypermobility syndrome	18	0:1	52	25-79
Normal	34	1:6	61	22-84

Table 6- Group 2 Effect of joint hypermobility on re-intervention following surgery for prolapse

Group	N	M:F	Median age	Range
Benign joint hypermobility syndrome	16	0:1	50	25- 78
Normal	26	1:6.5	58	23-84

All participants completed the follow up process; there were no deaths or drop outs to follow up.

2.1.3 Statistical analysis

Statistical analysis was performed using SPSS for windows. Age data was analysed using Student's T-Test and reoperation rates were analysed using Chi Squared Test with 2 degrees of freedom. A value of $p<0.05$ was deemed to be consistent with a statistically significant result.

2.2.1 Assessment of normal pelvic connective tissues

Normal pelvic tissue structural supports were analysed by a combination of macroscopic studies conducted primarily on cadaveric material and on microscopic analysis of pelvic connective tissues obtained from patients undergoing surgery for colorectal cancer. These patients were deemed to be the best patient group from which to take control tissue samples. This is due to the fact that it is possible to comprehensively assess their pelvic connective tissues during surgery. In addition the nature of the tissue sampling methods would make it unethical to take the tissues from a different patient group as it would necessitate a pelvic dissection which cannot be

condoned without clinical need. However, during cancer surgery for left sided, sigmoid and upper rectal lesions there is often a need to mobilise the proximal rectum and this enables tissue sampling from the area of interest to occur. Details of the assessment and sampling of the control tissues is provided in section 2.6.

2.2.2 Cadaveric tissue

Two whole formalin fixed cadavers were obtained (one of each sex)(Kindly donated by Professor J. Morris, Department of Anatomy). The ethics for cadaveric dissection was provided by a University held policy that allows for cadaveric dissection from those who have donated their bodies, voluntarily for medical research and anatomical study. They were free from any overt evidence of connective tissue disease and had not been diagnosed with pelvic organ prolapse nor undergone any previous abdominal or pelvic surgery. A full abdominal dissection was performed with careful dissection of the pelvic viscera. The pelvic organs were dissected in such a way that it was possible to identify the exact points of structural supports for the rectum. Mesorectal fat was removed by a combination of microdissection and xylene treatment to allow accurate visualisation of the structures which run through the fat layers and interdigitate with the more robust fascial condensations on the pelvic sidewalls. The abdominal phase of the dissection was concluded at the level of the mid rectum and then the cadavers were sectioned in the sagittal plane by sharp dissection. The pelvic viscera were also sectioned exactly in the sagittal plane. This then allowed more accurate study of the distal rectal supporting structures and their points of adhesion to the sphincter complexes.

2.2.3 Methods of tissue collection from patient s and controls

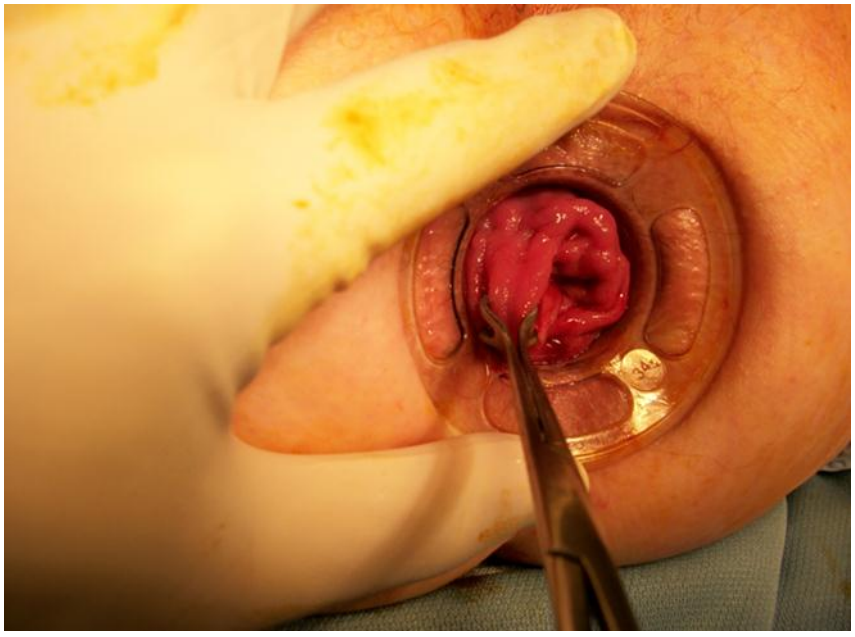
Patients

All participants had undergone a routine pelvic floor work up after presenting to the colorectal clinic. Presenting symptoms consisted of an externally palpable rectal prolapse, symptoms of obstructed defecation or chronic perineal / abdominal pain. The standard diagnostic work up is outlined below:

Table 7- Pelvic floor diagnostic work up

Test	Purpose
Colonoscopy	To exclude organic pathology such as colorectal cancer
Defecating proctogram	To identify occult internal prolapse or enterocele and help plan treatment
Colonic transit study	To try and identify sub population of patients with genuine slow transit constipation who may be better treated differently
Anorectal physiology studies	To evaluate the sphincter complex and determine sphincter function
Examination under anaesthesia using a Circular Anal Dilator (CAD)	Performed in patients with symptoms of obstructed defecation with non diagnostic proctograms. Conventional EUA may splint rectal prolapse and lead to an erroneously normal diagnosis. By using a CAD it is possible to determine with accuracy the site and extent of rectal prolapse.

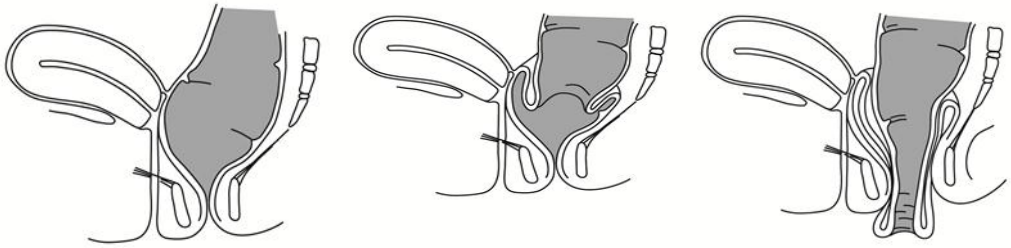
Figure 12 Examination of anus under anaesthesia using CAD.



This image shows a female patient undergoing an examination of the ano-rectum under general anaesthesia with full muscle relaxation. In order to fully demonstrate earlier degrees of rectal prolapse the use of a circular anal dilator can be useful. This clear plastic circular device splints the sphincter open, thereby allowing the prolapsing segment to be fully visualised. This particular case would be defined as stage 5 external rectal prolapse because it extends beyond the dentate line in a full thickness manner.

At the conclusion of these investigations a sub population of patients who present with the symptoms outlined above will be identified as suffering from internal or external rectal prolapse. These were considered eligible for entry to the study.

Figure 13- Oxford Prolapse Grading Scale



Normal appearances of rectum Internal rectal prolapse External rectal prolapse

Prolapse disorders can be objectively graded using the Oxford Prolapse Scale, The middle diagram is grade 3 and the right hand diagram is grade 5.

Participants were prospectively recruited. They were approached and invited to join the study and given a patient information leaflet. If they were happy to proceed then they were asked to sign a study consent form. A medical history was taken and a clinical examination to determine connective tissue status was also performed.

Surgery was performed under general anaesthesia with full muscle relaxation. A urinary catheter was inserted into the bladder. All study cases were performed via the laparoscopic route, this involved an infra umbilical incision and the abdomen was entered using a 10mm port. The abdomen was insufflated with carbon dioxide gas to maintain a pressure of 12mmHg.

Controls

Control tissue was taken from participants as described in section 2.8. The control group comprised patients who were due to undergo surgery to treat colorectal cancer. They were prospectively recruited and their connective tissue status was assessed as described in section 2.1.1.

The exclusion criteria were:

- Presence or history of rectal or pelvic organ prolapse
- Presence or history of connective tissue disorders
- Beighton score of >0
- Tumours of the rectosigmoid junction
- Any tumour that was either T3 or T4 on radiological assessment preoperatively or on clinical assessment intraoperatively
- Any active or recent pelvic sepsis or diverticulitis

The inclusion criteria were:

- Left sided colonic cancers with clinical stage of T1 or T2 that were scheduled for removal with a high anterior resection

A total of 12 control patients were recruited these comprised 10 males and 2 females, they had a median age of 73 years (range 56-79). The control group were typically older patients as these individuals are more likely to develop colorectal cancer (from which our control tissue was derived). It was important that they had not undergone any previous abdominal or rectal surgery. In practice males were more likely to fulfil this inclusion criterion as they are generally less likely to undergo surgery for pelvic floor disorders (which are relatively common in females).

2.3 Evaluation of pelvic and abdominal viscera in patients with prolapse

The pelvic and abdominal viscera were examined in all cases. As a result of multiple operative interventions several consistent findings were identified as being potentially associated with rectal prolapse and these were prospectively evaluated in a sub group of patients.

These observations were made prior to commencement of the surgical resection and included:

- Caecal pole located inferior to the lateral pelvic sidewall
- Peritoneal fat studding
- Isolated peritoneal defects
- Recession of the Pouch of Douglas below the cervix

The normal topography of the pelvic viscera is well described in anatomical texts and was verified by cadaveric dissection (see section 2.2.2).

2.4 Skin sample collection

The skin samples for the research were collected at this point and were taken from the right iliac fossa port site. In the initial phase of the research project, the tissue was excised using a knife or diathermy blade. Unfortunately, this resulted in considerable architectural disruption and precluded accurate assessment of the tissues. The technique was modified so that the tissues from the dermis were sampled using a 5mm dermatological punch biopsy. Samples were then bisected with half being snap frozen at -80^{o}C. The other half was preserved in 10% neutral buffered formal saline.

2.5 Pelvic connective tissue collection

The pelvic connective tissue sampling was performed as an intrinsic stage of the procedure of laparoscopic ventral mesh rectopexy. Using a diathermy device the dissection was commenced at the level of the sacral promontory, it was then continued down the right pelvic sidewall (figure 14). The right ureter and iliac vessels were identified and preserved. At the level of the peritoneal reflection the dissection was continued antero-inferiorly to the level of the anal sphincters, identified visually and by digital rectal examination. The dissection was completed by removal of the peritoneal reflection from the left pelvic side wall and the front of the rectum. This process yielded a segment of tissue (illustrated in figure 16). This was orientated; in this respect the sites of diathermy artefact were useful as they could allow for subsequent microscopic orientation. The centre of the specimen was usually free from any diathermy injury. Two samples were taken from this tissue, one portion was snap frozen to -80^{o}C and the other fixed in 10% neutral buffered formal saline.

Intraoperative tissue sampling from patients undergoing laparoscopic ventral mesh rectopexy

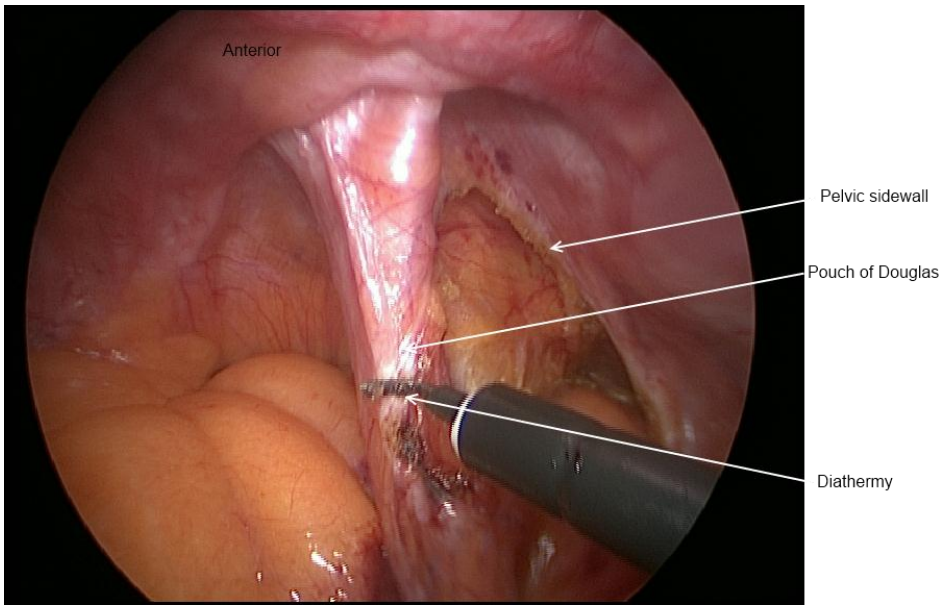


Figure 14 Anterior dissection of pelvic floor to expose rectum

Intra operative photograph taken during a laparoscopic rectopexy on a 73 year old multiparous female following a hysterectomy. At this stage of the procedure the right and side of the rectum has been mobilised and the dissection of the connective tissues anterior to the rectum is now taking place. On the left hand side of the image is the, as yet, unopened peritoneal reflection.

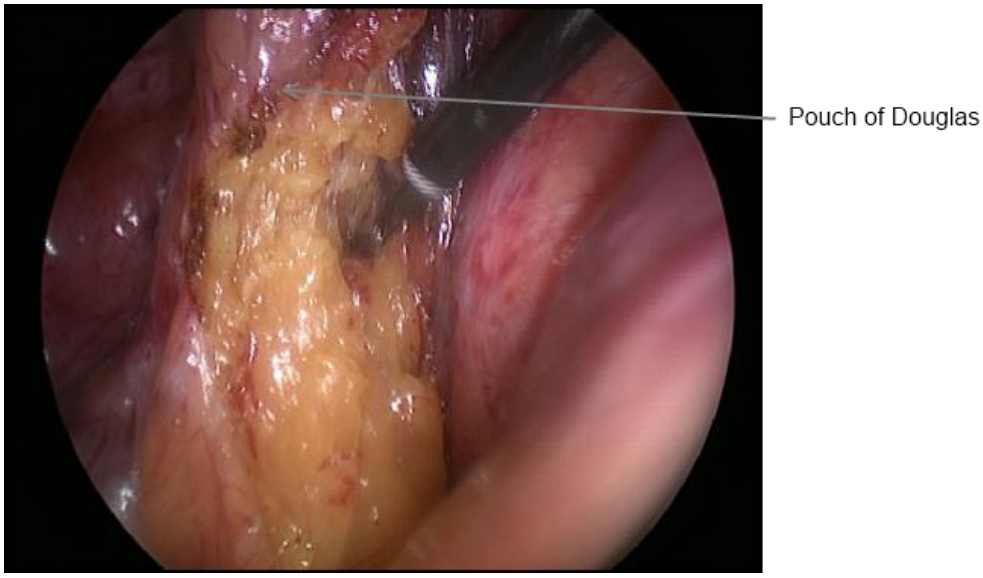


Figure 15 Surgical excision of Douglas Pouch

Intra operative photograph taken during a laparoscopic rectopexy from the same case as image 14. The rectal mobilisation is now completed and the connective tissues surrounding the anterolateral aspect of the rectum, together with the Pouch of Douglas, are being excised.

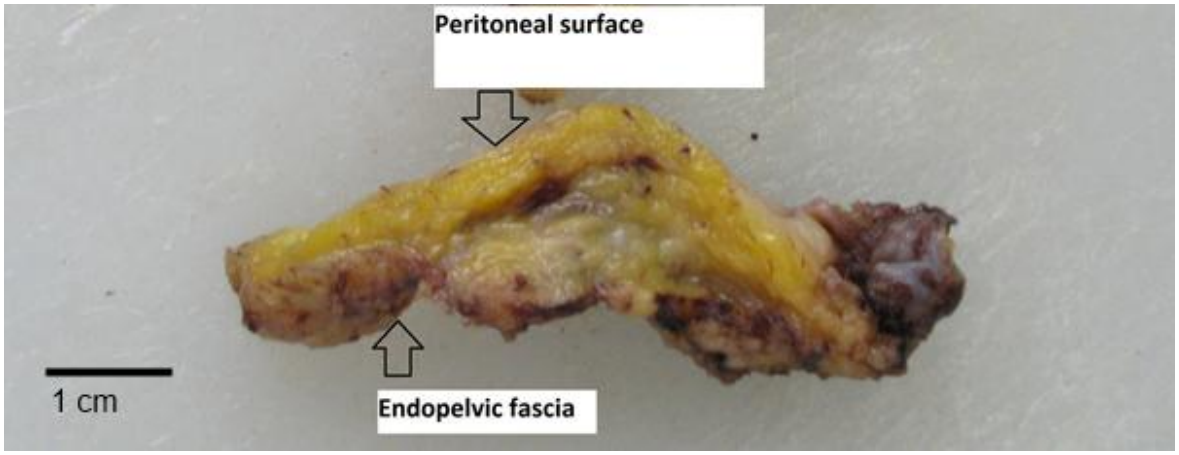


Figure 16 Pelvic floor connective tissue removed from patient

Photograph demonstrating the appearance of the tissues in figure 15 following removal from the patient and fixation in formalin. In this picture the tissues have been bisected in the sagittal plane and are thus viewed side on.

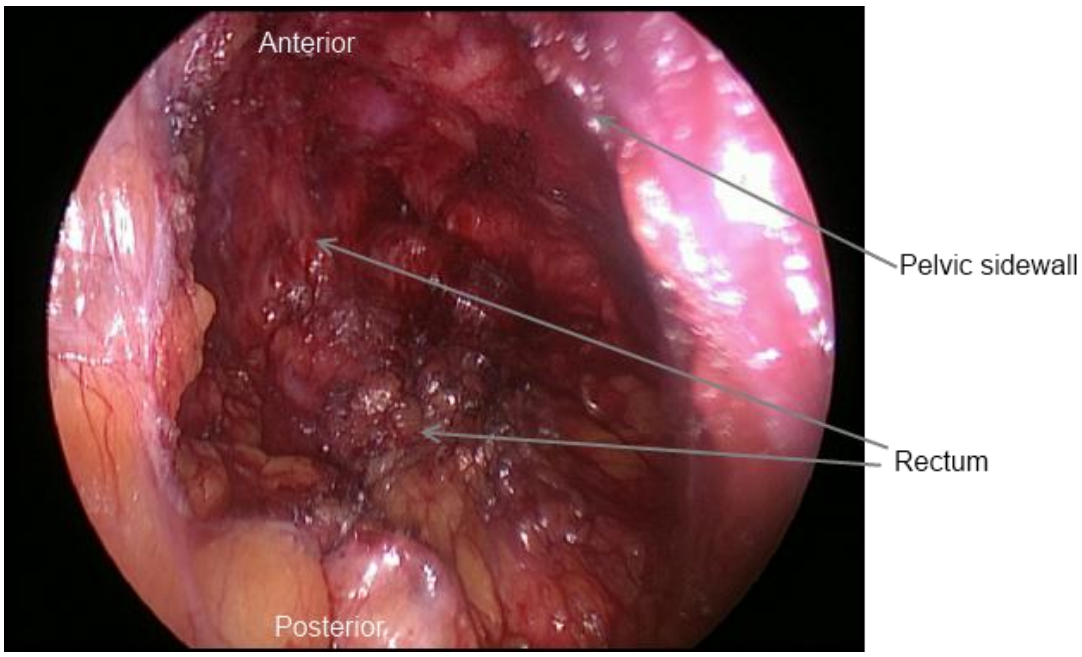


Figure 17 Defect created following rectal dissection and Douglas Pouch excision

Intra operative photograph from the same patient as image 14. The pelvic connective tissues have now been excised and the rectum mobilised. At this stage of the procedure the rectum will be restored to its standard anatomical position and a prosthetic mesh placed anterior to the rectum and fixed to the sacral promontory.

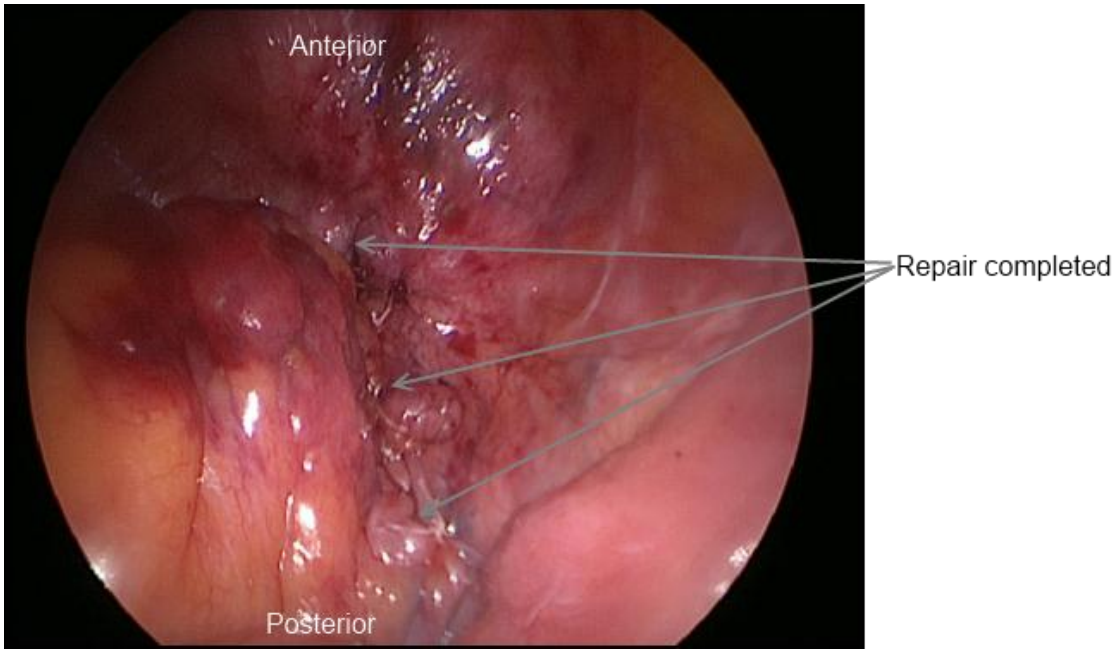


Figure 18 Site of surgery following sutured repair and mesh placement

In this intra operative photograph, from the same patient as image 14. The pelvic reconstruction is now complete and the pelvic floor continuity restored.

One of the main problems encountered with the tissue sampling were artefacts due to specimen handling and diathermy artefact. The issue of specimen handling relates to the need to surgically manipulate the tissues inside the patient. The use of a laparoscopic surgical technique results in the need to withdraw the tissues through a laparoscopic surgical port. This was only a problem with the Pouch of Douglas sampling in the symptomatic group. In order to avoid any excessive tissue damage during specimen removal the samples were removed through a 10mm laparoscopic port, these ports are fitted with a one way valve to prevent loss of pneumoperitoneum. Temporarily removal of this valve system enabled uncomplicated specimen extraction with minimal tissue trauma.

Surgical diathermy is a well recognised cause of iatrogenic damage during laparoscopic surgery and of potential cause of distortion of tissue architecture during histological assessment. Surgical diathermy is used extensively during the operations for both control and symptomatic patient tissues. Diathermy uses an electrical current to locally heat and vaporise tissues. It surgically

divides tissues whilst simultaneously providing haemostasis. The tissues adjacent to the surgical site will be heated and may be secondarily damaged as a result.

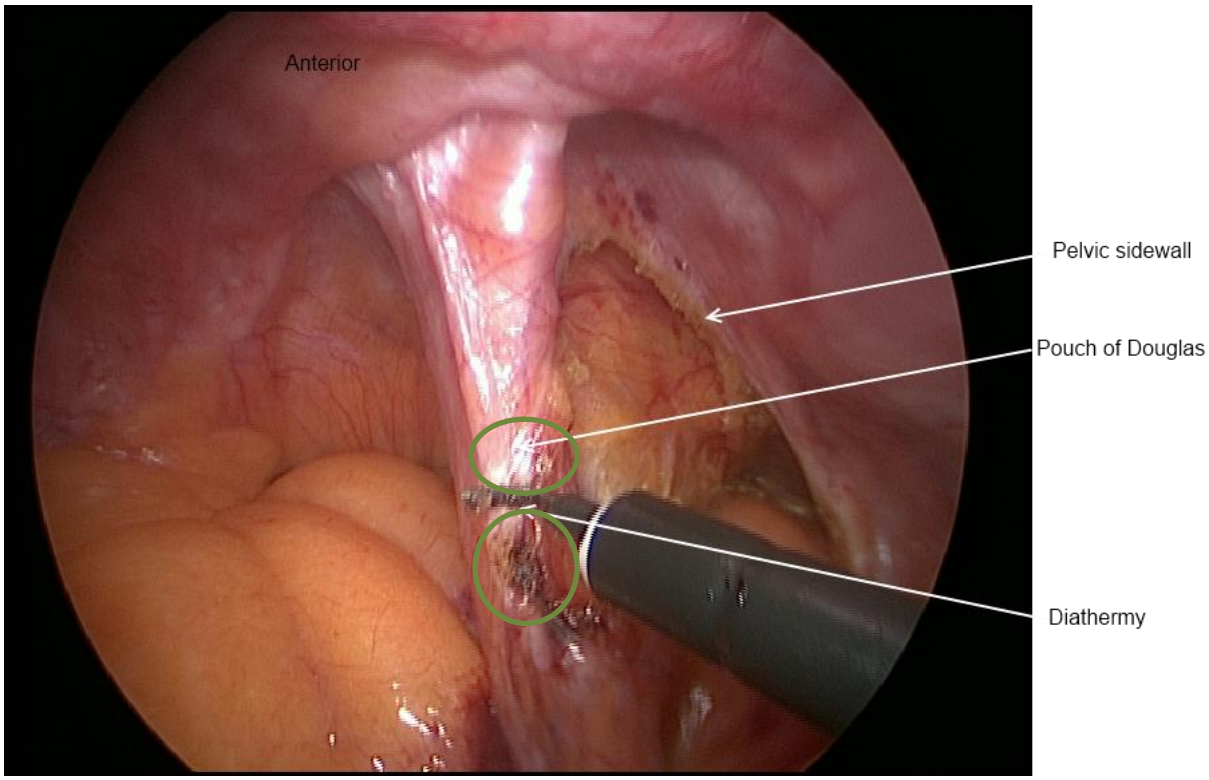


Figure 19 Diathermy effects on tissues intra-operatively

In this laparoscopic surgical image the effects of diathermy heating to tissues are demonstrated by the green circles. The tissues have a charred edge and white area adjacent to it demonstrating the burn extent.

The macroscopic effects of diathermy injury are obvious and to an extent unavoidable. Dissection of the tissue without the use of diathermy would result in excessive bleeding, this would in turn compromise both patient safety and, ultimately, the accuracy of tissue sampling. In the initial pilot studies the effects on tissues at a microscopic level were also evident, connective tissues were architecturally affected by the process. This makes it difficult to determine whether changes observed were the effect of the disease process or diathermy injury. Fortunately the effects of diathermy injury produce a series of characteristic histological changes, owing to protein denaturation (figure 20).

The sampling methods were adapted to take these effects into account. The characteristic macroscopic changes that occur as a result of diathermy enabled the tissue sampling to occur in a way that avoided these samples in tissue that was selected for detailed study.

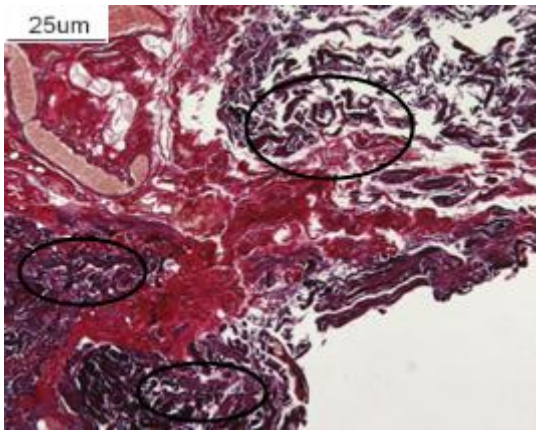


Figure 20- Microscopic effects of diathermy

This is a section of pelvic floor connective tissue stained using elastin van Gieson histological stain. It demonstrates the effects of surgical diathermy as outlined by the black circles.

2.6 Tissue processing

Following collection all samples were anonymised by allocation of a study number. This allowed easy transportation of samples without compromising personal patient details.

Tissue which was collected and snap frozen was transferred to the Department of Anatomy at the University of Oxford in dry ice and placed in a -80^{o}C freezer in sealed cryotubes. Prior to snap freezing it was cut into 2 x 2 cm sections.

Tissue in formal saline was left to fix for a minimum period of 48 hours. Once fixed, the tissues were removed and prepared for processing and embedding into wax blocks. On removal from formalin the pelvic connective tissue samples were cut in the sagittal plane such that the anterior and posterior aspects of the resection sites were included in the specimen. Where the specimen was large, it was transected at its mid point and both segments processed.

The samples were taken to the histology processing facility in the Dunn School of Pathology, University of Oxford. Processing was performed by a technician in the department (RS). The processing stages involve the substitution of water in the specimen for wax. Since these are immiscible the process involves graded exchanges of water for alcohol and then substitution of alcohol for xylene. Xylene is then substituted for wax and the samples may then be embedded.

The embedding phase was extremely important as it was crucial that the connective tissue samples were all embedded in the same plane. Provided that the specimens had been correctly orientated during the dissection phase this was not unduly complicated. Once the specimens were orientated they were immersed in molten wax and left to set.

For all light microscopy work sections were cut from the wax blocks by the pathology technician (RS) and placed onto vectabond coated slides(Vector Laboratories Ltd, Peterborough, UK). The use of coated slides was important in preventing specimen loss during the many antigen retrieval stages performed during immunohistochemistry. All sections were cut at 5µm.

2.7 Basic histological stains

2.7.1 Haematoxyllin and eosin

All samples were stained using haematoxylin and eosin, which demonstrates the organisation of tissues and cells, the technique is well described(127). In this project Harris Haematoxyllin was used and neutral eosin as a counterstain. This resulted in blue nuclei and pink connective tissues. Lipids would appear clear as they are removed during tissue processing.

2.7.2 Elastin van Gieson staining

This is a commonly used histological stain. The key stages are as below :

Table 8- Elastin Van Gieson Staining

Step	Rationale
Immersion in 0.5% potassium permanganate	Oxidation of elastic fibres
Washing step in water	
Immersion in 1% oxalic acid	Decolourise the potassium permangenate staining
Washing step in water	

Immersion in 100% ethanol	Subsequent stain is alcohol based
Immerse in Millers elastic stain (3 hours)	To visualise elastic fibres
Wash in 100% ethanol x3	Rinse off excess stain
Rehydrate sections to water	Subsequent stain is water based and decolourised by alcohol
Immerse in Van Gieson solution (10 minutes)	To stain collagen
Blot sections to remove excess stain	
Rapid sequence dehydration to xylene	Van Gieson is decolorised by alcohol and therefore minimal contact time is needed

A number of experiments were performed using this technique. The two most important steps in terms of maintaining specimen quality were the repeated washing of the section in alcohol after Millers elastic stain (Brunel Microscopy supplies), without which background tissue structures may appear excessively dark. The second is the careful blotting of the sections after the Van Gieson staining (Raymond Lamb microscopy supplies), otherwise excessive time must be spent dehydrating the slides with resultant loss of colour contrast. The colour contrast in this staining technique is of considerable importance because the image analysis methods used to quantify the surface area occupied by various tissue types rely on bold and clear staining of the tissue structures. Without clear contrast it is not possible to analyse the images.

2.7.3 Masson Trichrome staining

This was performed prior to the elastin Van Gieson method described above. It too is a stain for collagen fibres. However, it was not suitable for use with the elastic fibre stain and therefore serial sections were needed to correlate the two images. This coupled with the increased costs incurred as a result of this duplication led to this procedure being abandoned.

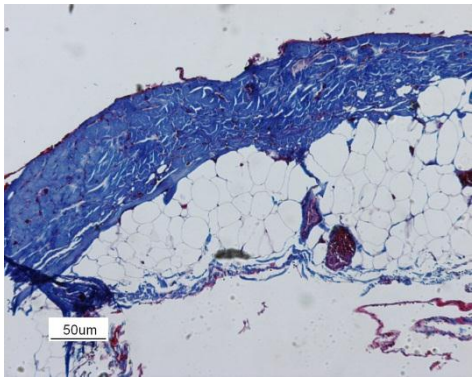


Figure 21 Pelvic floor connective tissue microscopy

Section of human pouch of Douglas sectioned in the Coronal plane and stained using Masson Trichrome

2.7.4 Glycosaminoglycan staining

Staining for GAG's was performed using a combination of alcian blue and nuclear fast red. This technique resulted in blue GAG's and red nuclei and other connective tissue elements. This staining technique required no special modification or optimisation.

2.8 Control tissue studies

Samples were obtained from patients who were undergoing surgery for colorectal cancer. To sample the tissues the distal resection margin included the upper mesorectum, the Pouch of Douglas or male equivalent was sampled and a sample of uninvolved rectal tissue was also taken. This tissue was either frozen at -80 degrees or fixed in 10% neutral buffered formal saline. Fixed tissue was processed histologically (as outlined in section 2.6). Staining with haematoxylin and eosin was performed (section 2.7) and elastin Van Gieson staining was also undertaken to allow more accurate visualisation of connective tissue structures (section 2.7).

In order to provide greater three dimensional resolution of the pelvic connective tissues selected samples were also subjected to multiphoton microscopy (section 2.16.2).

2.9.1 Immunohistochemical protocol

Many of the experiments conducted in this thesis rely on the process of immunohistochemistry. In this section an overview of the process of immunohistochemistry and the specific methods used in each of the subsequent experiments is provided.

Immunohistochemistry utilises the ability of monoclonal (or polyclonal antibodies) to identify foreign antigens. Antibodies can be raised in a host species by a process of selective antigen exposure and then appropriate antibodies produced and subsequently collected. In order to then visualise the antigen – antibody complex a secondary staining step has to be employed. In some cases it is beneficial to employ a process of amplification to produce an increased number of molecular binding sites for the staining molecules to bind to(128). The eventual staining solution

may be tagged with an immunofluorescent probe or a conventional dye that can be viewed using standard microscopic techniques.

In this project, harvested tissue was both frozen and fixed in neutral buffered formalin solutions. The former storage method has the advantage of keeping tissues in their native state. However, such tissue must be stored at -80^{o}C and since this storage space is limited the specimens themselves are smaller. The frozen tissue must then be cut into tissue sections using a cryostat. Here, problems were encountered in attempting to section the samples using a cryostat , these arose primarily by the variable tissue types present within the specimen, such as adipose and collagenous tissues of differing stiffnesses. Although the technique was eventually modified the resulting tissue gave extremely poor architectural resolution and was extremely wasteful of tissue. Moreover cutting frozen sections was extremely wasteful of time It was therefore decided to work with formalin fixed tissue as far as possible.

2.9.2 Fixed tissue antigen retrieval

The process by which the tissue samples were obtained and prepared to the wax block stage is detailed in section 2.6.

Formalin is a widely used histological fixative. The fixation of tissues involves the formation of multiple chemical cross links within the tissues themselves. This has the advantage of retaining architectural resolution. However, the cross linkage process may lead to masking of the antigen binding sites. Therefore it is necessary to unmask the antigen binding sites prior to performing the immunohistochemistry experiments. This involves selectively degrading the tissue cross links produced during the fixation process. This is a compromise situation because inefficient antigen retrieval will lead to insufficient antigen exposure(129). Overzealous antigen retrieval can induce false positive responses and / or lead to architectural damage to the tissues themselves. In general terms it is better to undertake antigen retrieval in a cautious manner and then continue with the immunohistochemical staining process. Because this may cause problems related to

steric hindrance with regard to subsequent binding of antigen-antibody staining, a number of procedures can be employed to optimise the antigen-antibody bonds for definitive stain. A commercially available kit is available for this purpose and was widely used. The avidin – biotin complex (ABC method(128)) was employed in most cases (Vectastain Elite ABC Reagents, Vector Laboratories Ltd, Peterborough, UK). A typical protocol for immunohistochemistry using the ABC method is provided below, together with explanations for each of the stages utilised.

Table 9- Immunohistochemistry protocol

Antigen retrieval process	To unmask antigen binding sites if required
Incubation of samples with phosphate buffered saline solution	To wash all antigen retrieval solutions from specimen
Incubation with 0.3% hydrogen peroxide solution for 20 minutes	The final staining step with DAB stain utilises a peroxidase reaction. This step will eliminate all endogenous peroxidase activity.
Incubation with phosphate buffered saline solution	To wash the hydrogen peroxide from the sections
Incubation of sections with 1% normal horse serum	All primary antibodies were raised in mice. In order to minimise false positive staining the slides were immersed in sera from a different species to maximise specific tissue binding.
Incubation with primary antibody	To allow antigen – antibody binding
Incubation with PBS	To wash all unbound primary antibody from the section
Incubation with biotynilated antimouse antibody	This allows binding of the biotin molecule to the antigen (human)- antibody(mouse) complex
Wash with PBS	To wash unbound biotynylated antimouse antibody from the sections
Incubation with avidin	Avidin has a high affinity to biotin and this then forms a avidin – biotin molecular complex
Wash with PBS	To rinse unbound avidin from the section
Incubate with definitive tissue stain e.g.DAB	To visualise the avidin – biotin –antigen-antibody complex
Counterstain	To visualise non stained tissue structures.

2.9.3 Immunohistochemical methods

In the table below are outlined the specific methods for each of the immunohistochemical methods employed in the research. The microwave techniques used were a combination of

methods in the published literature(129)and the standard operating procedures manuals at the Oxford Radcliffe Hospitals NHS trust (Kindly donated by Professor Warren).

Table 10- Immunohistochemistry antibodies and methods

Experiment	Antibody used	Antigen retrieval method	Secondary antigen amplification steps	Staining method
Collagen 1	Monoclonal mouse anti human collagen 1 (Dilution 1:50) (AdB Serotec, Kidlington, UK)	Enzymic digestion with 1% buffered trypsin	ABC method	DAB with nuclear fast red counterstain
Collagen 3	Monoclonal mouse anti human collagen 3 (Dilution 1:50) (AdB Serotec, Kidlington, UK)	Enzymic digestion with 1% buffered trypsin	ABC method	DAB with nuclear fast red counterstain
Cellular proliferation	Monoclonal mouse anti human Ki67 protein (Dilution 1:100) (DAKO UK Ltd, Ely, UK)	Microwave with DAKO target retrieval solution pH6	ABC method	DAB with nuclear fast red counterstain
Myofibroblast	Monoclonal mouse anti human smooth muscle actin (for fibroblasts) (Dilution 1:100) Secondary antibody incorporated into DAPI counterstain (DAKO UK Ltd, Ely, UK)	Microwave with DAKO target retrieval solution pH 6	No	Dylight 460 sheep anti mouse fluorescent dye with DAPI counterstain
Transforming growth factor β	Monoclonal mouse anti human TGF β (Dilution 1:70)	Not required	ABC method	DAB with nuclear fast red counterstain
Oestrogen	Monoclonal	Microwave with	ABC method	DAB with methyl

receptors	mouse anti human oestrogen receptor alpha (Dilution 1:100) (DAKO UK LTd, Ely, UK)	DAKO target retrieval solution pH6		green counterstain
TIMP 1	Monoclonal mouse anti human TIMP 1 (Dilution 1:100) (DAKO UK Ltd, Ely, UK)	Microwave with DAKO target retrieval solution pH6	ABC method	DAB with nuclear fast red counterstain
MMP 1	Monoclonal mouse anti human MMP1 (Dilution 1:50) (ABCAM, Cambridge, UK)	Microwave with DAKO target retrieval solution pH6	ABC method	DAB with nuclear fast red counterstain
MMP 3	Monoclonal mouse anti human MMP3 (Dilution 1:50) (ABCAM, Cambridge, UK)	Microwave with DAKO target retrieval solution pH9	ABC method	DAB with nuclear fast red counterstain
MMP 7	Monoclonal mouse anti human MMP7 (Dilution 1:100) (ABCAM, Cambridge, UK)	Microwave with DAKO target retrieval solution pH 9	ABC method	DAB with nuclear fast red counterstain
MMP 9	Monoclonal mouse anti human MMP 9 (Dilution 1:100) (ABCAM, Cambridge, UK)	Microwave with DAKO target retrieval solution pH 6	ABC method	DAB with nuclear fast red counterstain

Elastin was not assessed using immunohistochemical techniques, there were two reasons for this. Firstly, the basic elastic fibre stain that was used gave clear, reproducible staining and was considerably more cost effective than a more complex immunohistochemical stain. Secondly, the antigen retrieval process required for elastic fibre visualisation would have been inherently destructive to the tissues, compromising the work which was planned.

2.10.1 Cellularity studies

Preliminary cellularity studies were conducted on formalin fixed, paraffin embedded tissues stained with haematoxylin and eosin. The samples were taken from the pelvic connective tissues of patients undergoing surgery for rectal prolapse as described in section 2.5. Slides were viewed at low power magnification and the regions of maximal cellularity identified. From these regions high power images were viewed and the cells present in the extracellular matrix were manually counted and expressed as total cells per high power field. Endothelial cells were not included in these assessments. Results were analysed according to the patient groups outlined in section 5.1 using SPSS for windows and a Students T Test was performed. A level of $P<0.05$ was deemed to be consistent with a significant result.

2.10.2 Assessment of cellular proliferation

Analysis of cellularity between the patient sub groups demonstrated differences. To determine whether this represented a basal situation or a potential response to the process of rectal prolapse the cells were studied to determine whether they were proliferating. This was performed using immunohistochemistry with anti Ki67 antibody (method as outlined in section 2.9.3). Ki67 is a nuclear protein that is expressed in the nucleus from the chromosomal surface in all phases of mitosis, with the exception of G_0 and will therefore accurately identify those cells that either actively proliferating or preparing to do so(130). Once stained, slides were viewed and the areas of maximal cellular proliferation were counted in each high power field. . Results were expressed as percentage cells per high power field and analysed using SPSS for Windows a Students T Test was performed and P value of <0.05 was deemed to be consistent with a significant result.

2.10.3 Cellular differentiation

As stated previously this involved determining whether the fibroblasts had differentiated to a myofibroblast subtype. In order to study this most effectively it was felt that use of immunofluorescence would be the most efficacious method. The detailed immunohistochemical

method is provided in section 2.7. Image acquisition was performed by studying the sections using two different UV light wavelengths. This allowed the visualisation of both nuclei and smooth muscle fibres. These images were obtained from the same geographical region of the specimen. The images were saved as tiff files and then image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012) was used for image reconstruction. The two separate images were fused and could thus be viewed as a whole composite image. Manual cell counts of the number of myofibroblasts present in high power section could then be undertaken. Data was analysed using SPSS for Widows and a Students T Test was performed, a P value of <0.05 was deemed to be consistent with a significant result.

2.11.1 Matrix metalloproteinases in pelvic connective tissues

In this study MMP's 1,3,7 and 9 were evaluated. These were primarily targeted as MMP 1 is capable of cleaving intact collagen, whilst the other members of the group are implicated in degrading part denatured collagen (see section 1.6). Their endogenous inhibitor, TIMP 1, was also evaluated.

In the table below are the patient characteristics for the sub group that were analysed for MMP and TIMP distribution.

Table 11- MMP patient characteristics

Group	N	Mean age	Range
Control	4	68	50-75
Nulliparous women	6	32	22-59
Multiparous women	18	52	23-70
Males	5	51	22-66

Experiments were conducted sequentially, i.e. they were not all conducted on the same slide with multiple counterstains. It was felt that this technique could lead to interpretation problems. The slides were prepared and stained as outlined in section 2.6. Following staining, slides were

reviewed by a consultant histopathologist (B. Warren) to ensure staining consistency. Slides were graded according to intensity of staining(129). In addition the number of cells which stained positive were counted. The grading system employed is described below:

Table 12- Immunohistochemical grading

Grade 0	No positive staining.
Grade 1	Weakly positive staining.
Grade 2	Moderately positive staining, with at least one area of strongly positive staining that accounts for less than 50% of total section area.
Grade 3	Strongly positive staining which occupies a total of >50% of the total section area.

In the case of the pelvic connective tissue specimens this system was applied to the section as a whole.

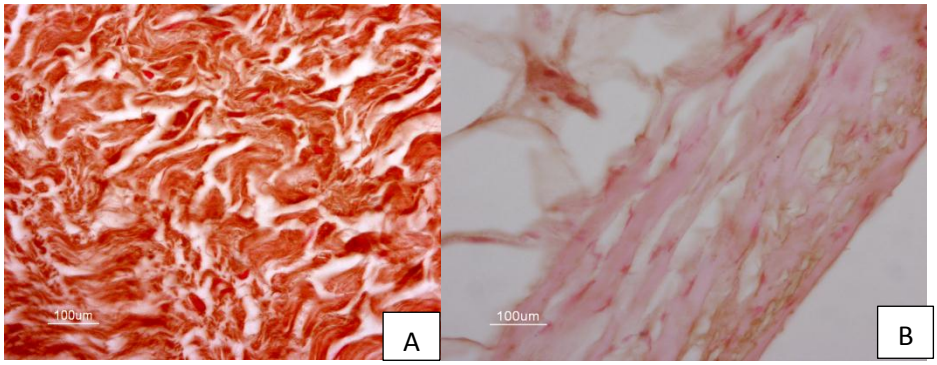


Figure 22 – Demonstration of grading examples

Sections of pelvic connective tissues stained for MMP1, section A demonstrates grade 3 staining and section B demonstrates grade 1 staining.

Since MMP 1 is a collagenase, the relationship between staining intensity and fascial band thickness was assessed. The patients with all 3 grades of MMP 1 staining then had their fascial

bands assessed using a different staining technique (since the use of microwave may have disrupted them). The bands were sub divided into 3 groups; absent, thin and thick. Comparisons were then made between the groups. The intention of this study was to be purely descriptive in terms of the distribution and approximate concentrations of MMP's that were present, as such, it was not intended to conduct formal statistical analysis of this data.

2.12.1 Assessment of basic fibroblast growth factor activity

Immunohistochemistry was used to identify bFGF in the pelvic connective tissues of patients with rectal prolapse (details given in section 2.9). Once the slides were stained the sections were viewed microscopically at both low and high power magnification. Digitised images were created and saved as tiff files. These were uploaded onto the image J image processing system. A technique of subtraction thresholding (figure 23 section 2.17.5) was used to remove all areas of the tissue that did not stain positively for bFGF. The area occupied by the positive tissue and cells was then calculated. As there is a generalised lack of knowledge about bFGF in pelvic connective tissues the experiments were conducted primarily to identify the distribution patterns and staining intensity for bFGF. Because the image analysis software is able to provide semi qualitative data, it was decided to analyse this in a post hoc manner using SPSS for windows, both ANOVA and LSD post hoc testing was performed and a P value of less than 0.05 was deemed to be consistent with a significant result.

2.13 Transforming growth factor β

Transforming growth factor β was studied using immunohistochemistry. Sections were processed as detailed in sections 2.4 and 2.7. Stained sections were viewed using a standard light microscope. It soon became apparent that TGF β staining was localised to both an intracellular and pericellular location and therefore it was decided to quantify this using image analysis methods. Digitised images were created and uploaded onto the Image J system. Positive thresholding was used to delineate and subsequently calculate the area of positivity as a percentage of the total section size. Because the image analysis software is able to provide semi

qualitative data, it was decided to analyse this in a post hoc manner using SPSS for windows, both ANOVA and LSD post hoc testing was performed and a P value of less than 0.05 was deemed to be consistent with a significant result.

2.14 Oestrogen Receptors

Oestrogen receptor status was determined using immunohistochemistry. The main elements of the staining process are described in section 2.7.

Table 13- Patient characteristics for oestrogen receptor study

Group	N	Median age (range)
Control	4	63 (57-65)
Nulliparous women	5	23 (22-33)
Multiparous women	14	63 (40-74)
Men	6	48 (23-64)

Sections were viewed using standard light microscopy. The method of oestrogen receptor assessment was originally designed for use in the management of invasive breast cancer. This technique was used in this study and gives a figure, the H Score, which reflects oestrogen receptor positivity(131).

The method involves viewing the sections and determining the percentage of cells which are positive for oestrogen receptor expression, these are classed as showing weak, moderate or strong. To score the section, the strength of staining intensity is weighted such that weak staining is multiplied by 1, moderate by 2 and strong by 3. The total figure obtained for the entire section is referred to as the H score. In treating breast cancer the scores are interpreted as follows:

Table 14- Oestrogen receptor scoring

Score	Result
0-9	Negative
10-49	Weakly positive
50-300	Strongly positive

These are weighted in that clinical setting to ensure that women with oestrogen receptor tumours are not denied hormonal therapies. It was decided to use this result system in this trial, accepting that it was validated in a different disease process. It was decided to analyse the oestrogen receptor score data using SPSS for Windows with Students T Tests, a P value of <0.05 was viewed as being consistent with a significant result.

2.15 Collagen 1 and 3 studies

To determine the distribution patterns and relative intensities of collagen 1 and 3 in pelvic connective tissues immunohistochemistry was performed. The protocol which was eventually followed is outlined in sections 2.4 and 2.7. However, the collagen studies posed a considerable problem. This was most marked with collagen 1, the collagen 3 antibody gave good reproducible staining. However, the collagen 1 required trialling of multiple antigen retrieval methods and concentration dilutions in order to optimise staining. The use of microwave gave the best antigen retrieval. However, the architectural disruption that resulted rendered the sections un-interpretable. Eventually the enzymic methods of antigen retrieval and use of a protocol kindly donated by Professor S. Roberts (Robert Jones and Agnes Hunt Hospital, Oswestry, Appendix 2), resolved the problem.

Sections were jointly viewed with a pathologist (BW) and it was felt that a grading system would be the best method of describing the distribution patterns that were seen.

Table 15- Immunohistochemical grading for collagen

Grade	Staining characteristics
Grade 1	Weakly positive staining
Grade 2	Moderately positive staining, with at least one area of strongly positive staining that accounts for less than 50% of total section area.
Grade 3	Strongly positive staining which occupies a total of >50% of the total section area.

In this case absent staining is excluded from the classification. This is because it is the sub peritoneal collagen layer that is being specifically evaluated. It would be most unusual for staining to be completely absent in this situation as complete absence of collagen 1 is generally a lethal phenotype not compatible with life. The results for collagen 1 and 3 are presented jointly. This is because previous research interests have focussed on the relative proportions of these two collagen subtypes in pre-disposing to prolapse disorders.

Other techniques for evaluating total collagen content of the connective tissues.

We used hydroxyproline assays, a biochemical method which involves digesting the tissue sample (132)The tissue types are of mixed type including fat, connective tissues and regions of high cellularity, all of which have a variable quantity of collagen present (see section 3.2)It is thus difficult to sample identical areas from each sample on each occasion and variable results were obtained and were not reproducible from patient to patient. It was initially planned to follow up the hydroxyproline quantitation collagen by cyanogen bromide digestion which allows quantification of the collagen subtypes present through separating them on a gel (133). As even a relatively simple technique like the hydroxyproline assay could not give useful information patient to patient, immunohistology appears to represent the best compromise method for assessing these collagen types.

Results were analysed by the patient groups as described in table 29 (Chapter 8) as it was felt that any connective tissue abnormalities would most likely be present in those groups that had least in the way of mechanical pelvic floor trauma. The decision was made to describe the findings and relative proportions of collagen types that were present and not to conduct statistical analysis on the data.

2.16 Multiphoton microscopy

Multiphoton microscopy was performed as a means of further investigating the connective tissues present in the pelvis. This procedure is performed on fresh tissue that is thicker than those samples studied using light microscopy and therefore is able to provide better three dimensional resolution of the connective tissue arrangements.

1.16.1 Background and rationale

Conventional microscopy relies on the ability of light to pass through a tissue section contained on a glass slide. Light passes through the tissues and it then visualised by the naked eye (usually through a magnification lens). The structures in that tissue may be stained by artificial dyes that will absorb photons of a certain wavelength from that section that will then allow them to appear a certain colour. In order to perform this process with a conventional microscope, tissues need to be processed (section 2.4). Light microscopy therefore has a series of potential drawbacks including the inability to generate three dimensional tissue images (unless a very large number of sections are cut). There may also be concerns that the tissues themselves may be altered by the processing method and that therefore changes identified on conventional microscopy may be the result of artefact.

Multiphoton microscopy is able to generate images because certain materials possess the ability to fluoresce when excited by energy of a certain wavelength. Fluorescence occurs when an electron is promoted to a higher energy state and then falls back to its native state, emitting energy as light as it does so. This light can be captured using a camera and then processed. In this project the predominant tissues of interest in supporting the rectum are fat, collagen and elastic fibres. These all contain the ability to fluoresce if excited by light of an appropriate wavelength. This technique avoids the need to pre treat the tissue with any exogenous dyes or chemicals. It also allows study of thicker samples of tissues because the light is projected onto and emitted from the specimen, an important difference between multiphoton and conventional microscopy. There is a limit to the thickness of biological samples that can be studied because very thick

pieces of tissue will require higher laser energy levels to penetrate the specimen, a process that can damage biological tissues and induce artefact.

2.16.2 Protocol for multiphoton microscopy

Table 16 – Multiphoton microscopy protocol.

Process	Purpose
Snap freezing of tissue to -80°C	Preserve tissue in native state
Thawing of tissue to 18°C	To allow specimen preparation
Cutting tissue to 3mm thickness	To allow light source to adequately penetrate the specimen
Immersion of specimen in 0.9% sodium chloride	To ensure osmotic neutrality and prevent cellular distortion
Specimen orientation using brightfield microscopy	To ensure that specimen is orientated correctly prior to energising the lasers
First laser scanning using two photon fluorescence set at 0.90 milliwatts with spectral filter 5 deployed	To provide visualisation of elastic fibres within the specimen
Second laser scanning using single photon laser SHG set at 0.90 milliwatts with spectral filter 6 deployed	To provide visualisation of collagen fibres within the specimen
Third laser scanning using twin photon (TPF) laser set at 270 milliwatts with spectral filter 1 deployed	To visualise the adipocytes within the specimen
All three data files saved as tiff files types	To maximise amount of data captured
Image stacking and channel merging using image J	To integrate the three separate data files and allow topographical study of the three separate structural elements

2.17.1 Elastic fibre analysis in pelvic connective tissues and skin

Elastic fibres were assessed in both skin samples and pelvic connective tissues of patients with rectal prolapse. The skin samples were taken to determine whether changes observed in the pelvic connective tissues were simply the result of the disease process or part of a multisystem disorder. The samples were collected as described in section 2.5. A small subset of pelvic connective tissues were also subjected to multiphoton microscopy for a separate analysis of elastic fibre distribution and morphology as described in section 2.16. Once collected, the skin and pelvic connective tissue samples were processed as described below.

Table 17- Elastic fibre patient characteristics

Patient group	Average age	Number
Control	68.0	4
Males	53.0	8
Nulliparous women	32.8	9
Multiparous women (with internal prolapse)	35.3	24
Multiparous women (with external prolapse)	61.7	7

2.17.2 Correlation between dermal and pelvic connective tissue elastic fibres

Paired samples from the groups described above (table 17) were compared. The percentage of elastic fibres in each tissue type was determined as described in figure 23. Linear regression analysis was performed using SPSS for Windows and an R^2 value of >0.9 was deemed to indicate a significant correlation between with two groups. To attempt to mitigate the effect of the disease process the data for the control group was analysed separately.

2.17.3 Relationship between pelvic elastic fibres and joint hypermobility syndrome

A sub population from the elastic fibre groups (described in table 17), were analysed. They were clinically assessed to determine their connective tissue status as described in section 2.1. The demographics of this population are given in table 17.

Table 18- Demographics of elastic fibre sub groups tissue mobility status

Group	N	Connective tissue status
Normal	10	Beighton score 0
Clinically minor features of hypermobility	6	Beighton score 1-3
Benign joint hypermobility syndrome	7	Beighton score >3 (+features of BJHS)

Statistical analysis was performed using SPSS, with ANOVA and LSD post hoc testing, a p value of <0.05 was deemed be consistent with a significant result.

2.17.4 Tissue staining methods for identification of elastic fibres

5µm thick sections were cut and placed on vectabond coated slides. They were dewaxed through xylene and graded concentrations of alcohol to complete rehydration. Thereafter the method described in section 2.7 was used.

2.17.5 Specimen analysis for elastic fibres

Slides were viewed using a light microscope. The stain used resulted in crisp, clear and even staining with specific visualisation of elastic fibres. To assess the area occupied by elastic fibres the collagenous layer of the pelvic connective tissues and the dermis of the skin biopsies were viewed at high power and digitised images captured using the ACT 1 image capture system (NIKON Microscopy systems Japan) and saved as tiff files. These were uploaded to image J and thresholded to exclude the collagen and then calculate the area occupied by elastic fibres. In a manner similar to that previously utilised by others(49, 134).

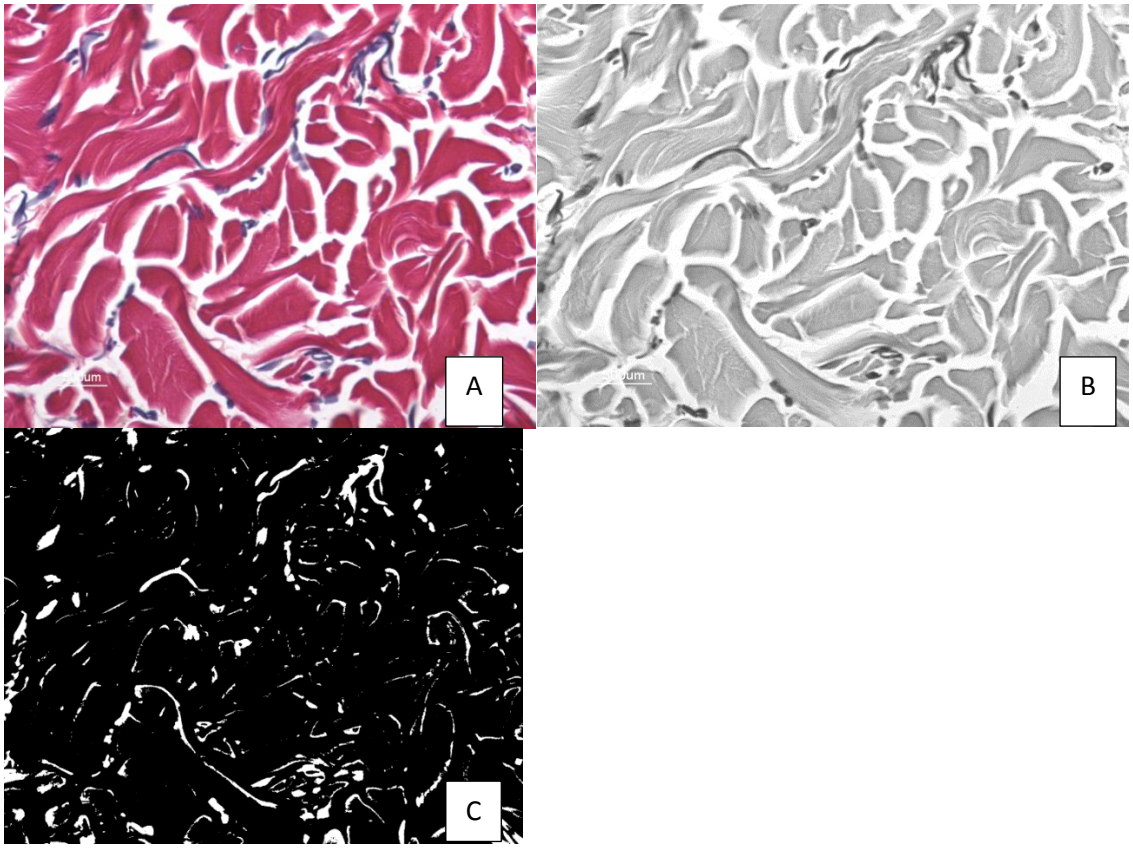


Figure 23 Image J Thresholding process for elastic fibre quantification

Series of images constructed using Image J for analysis. In picture A is featured a photomicrograph of human dermal tissue stained using the elastin Van Gieson technique described in section 2.7.2. Picture B demonstrates the effects of colour conversion to grayscale. Once the image is converted to greyscale, it can then be inverted to demonstrate the black fibres (image C). The process of thresholding defines the greyscale intensity that will be selected. Fibres that may not immediately be apparent with the naked eye are detected using this process and this increases the accuracy of the subsequent analysis. It is for this latter reason that high intensity data files are used for image capture (TIFF files). The use of compressed file formats such as JPEG or bitmap images does not capture sufficient data to enable this level of image processing and would result in blurred images that cannot be analysed.

Chapter 3

Results

3.1 Normal pelvic connective tissues and colon

3.1.1 Macroscopic structure

The macroscopic structures were well visualised by the naked eye. Much is already known about the fascial planes surrounding the rectum because of their importance in cancer surgery. However, because of the nature of cancer surgery the structures that facilitate adhesion between the rectum and the fascial condensations are not so well understood.

In the normal human rectum there are two well described condensations of the endopelvic fascia surrounding the rectum, anteriorly lies Denonvilliers fascia and posteriorly lies Waldeyers fascia(15). In females there are additional fascial condensations surrounding the uterus and these too will blend with the endopelvic fascia(15).

Topography of the connective tissue network

The rectum itself is encased by the mesorectal fat; superiorly this is continuous with the colonic mesentery superior to the recto-sigmoid junction. At the level of the peritoneal reflection the peritoneal surface spans out across the pelvic floor towards the lateral pelvic sidewalls (see figure 20).

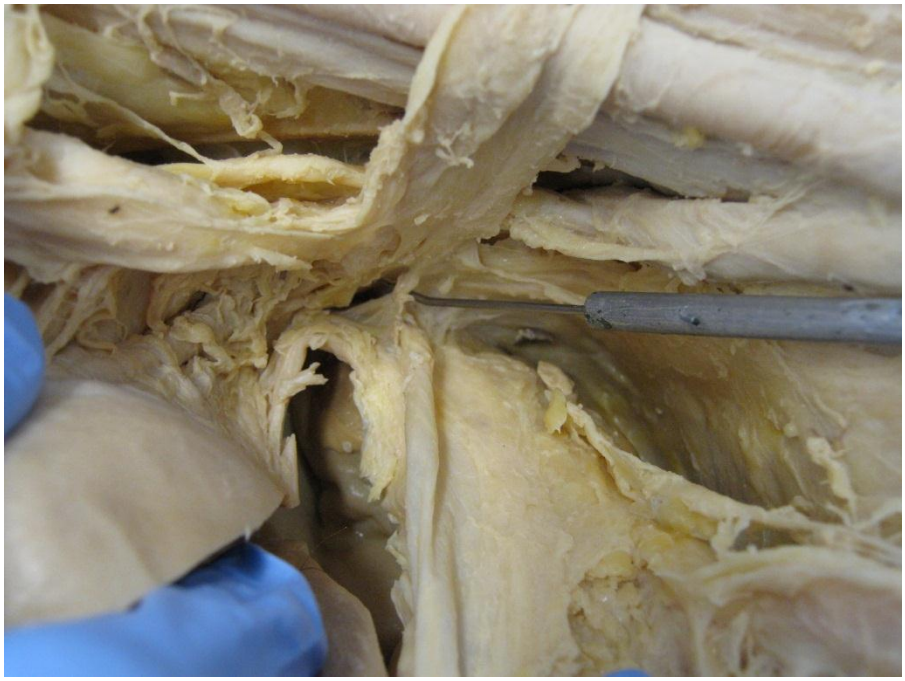


Figure 24 Endopelvic fascial condensations of the pelvic sidewall

Human cadaveric dissection from female cadaver. The image shows a close up image of the right extraperitoneal pelvic sidewall. The pointer indicates the point of attachment of the endopelvic fascia with the uterosacral ligaments.

The deepest point of the peritoneal cavity is the recess that lies between the rectum and uterus (the pouch of Douglas). During the cadaveric dissections (methods described in section2.2.2) and from my own operative experiences it is apparent that there is not a simple transition between peritoneum and fat. Aside from the fascial condensations both anteriorly and posteriorly there also lies a collagenous support layer that runs under the surface of the pelvic peritoneum.

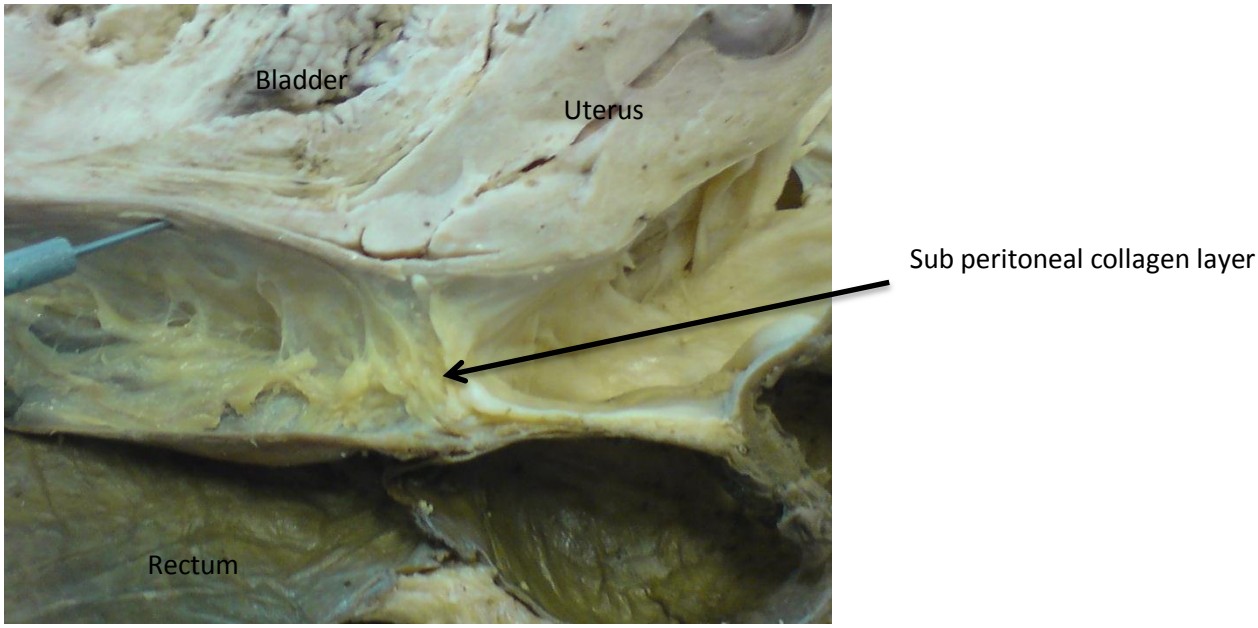


Figure 25 Tissues surrounding the rectovaginal septum

Cadaveric dissection, cadaver sectioned in the sagittal plane. At the lower part of the picture lies the rectum. At the uppermost aspect lie the uterus and bladder. The fat has been partially cleared and the fibrous tissue interspersed between it, the rectovaginal septum and pouch of Douglas is demonstrated.

This is in direct continuity with the endopelvic fascia and thus potentially a continuation of the supporting role it plays. This layer is not thick (normally 150µm), although in some patients with rectal prolapse it may become thickened (up to 800µm). More inferiorly and running throughout the mesorectal fat are small blood vessels and fascial condensations (called fascial bands in this thesis).

3.1.2 Rectal anatomy

The normal rectum lies within the mesorectal fat and gently follows the inclination of the sacrum and coccyx towards the pelvic floor. Distally the mesorectum tapers and the sphincter complex surrounds the distal rectum and anus. The dissection work undertaken confirmed that the endopelvic fascia is continuous with the sphincter complex. Inside the rectum the mucosal surface is marked by a number of villous structures which lie on the basement of the lamina propria. The normal colonic crypt is an elongated structure and the lamina propria not overtly

thickened. In the normal situation elastic fibres are not seen to any great extent within the mucosal layer. The distal extent of the rectum is marked by the dentate line and in the normal situation rectal mucosa should not project beyond it.

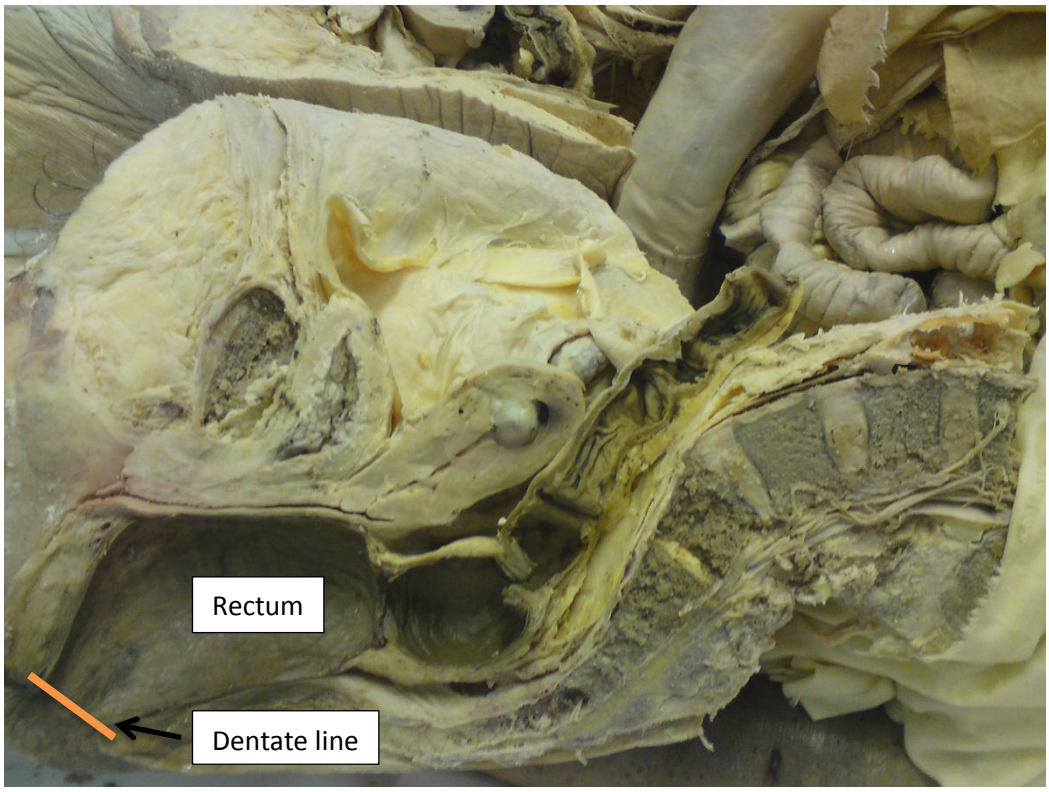


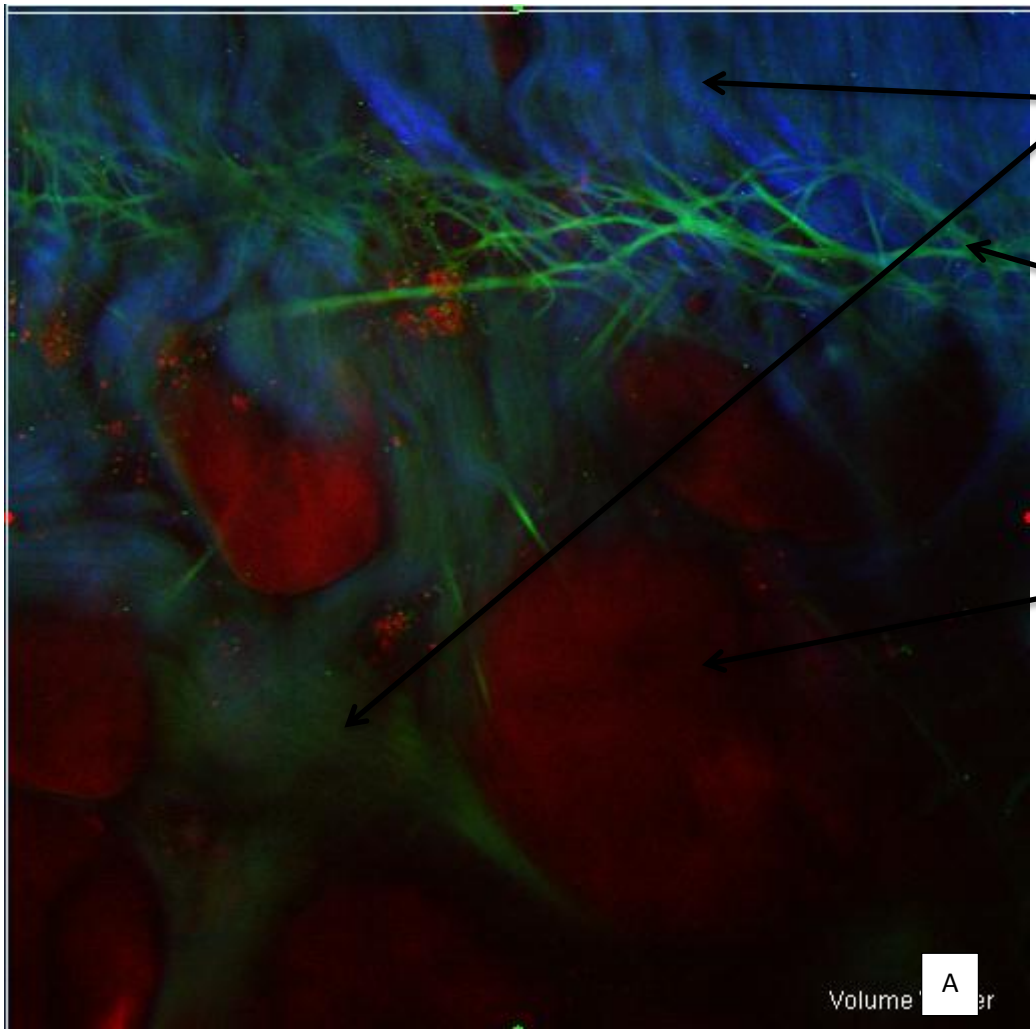
Figure 26 – Human pelvic anatomical topography

Human cadaver (female) sectioned in the sagittal plane. The rectum should not protrude below the dentate line (marked in orange). External rectal prolapse occurs when the rectum protrudes beyond this level.

3.1.3 Microscopic normal anatomy

At the level of the peritoneal reflection (see figure 27) the sub peritoneal collagenous layer adopts a lattice type arrangement. Fibres from this layer project down through the fatty tissue to interdigitate with the fascial bands running through the mesorectal fatty layer so that the entire rectum is encased within a complex collagen mesh. In the normal situation these layers are not excessively thick and in some cases may just be 50-100µm wide. It is important to appreciate that in the normal situation this layer is both distinct and continuous and collagen fibres are clearly

identifiable (as illustrated in figure 27). Located within this collagen network are upward projections of an elastic lattice arrangement that lies at the interface between the collagenous layer and the fat.



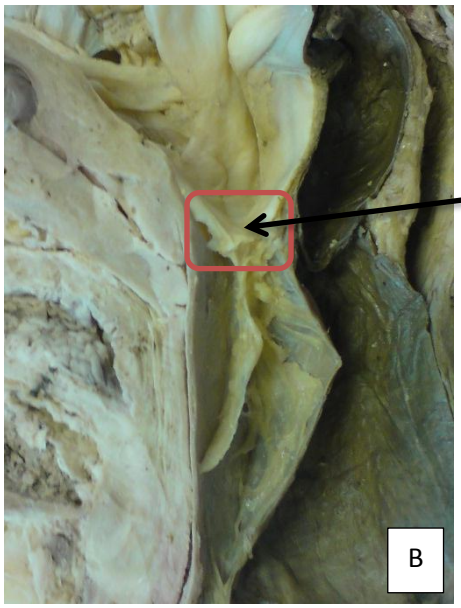


Figure 27 Pelvic connective tissue structures

Image A is taken from a cross section of pelvic connective tissue harvested from a female with rectal cancer at the time of anterior resection. In picture B is an orientation image taken from a sagitally sectioned human cadaver, with the sampling region delineated by the red square (scale 1cm=25µm). The multiphoton images are processed using the Image J system to produce colours to facilitate demonstration of the relevant structures within the pelvic connective tissues (elastic fibres – green, collagen- blue and fat – red). The lattice like arrangement of the pelvic elastic fibres (stained green) at the level of the peritoneal reflection is well demonstrated in this image and indicates the structural integrity of this layer.

The elastic layer itself is wide and consists of a flatter tissue structure with a distinct overall shape (see figure 27). Single fibre projections run from this layer to the fat layer beneath and the collagen layer above. In most cases the elastic fibres are usually co-localised to collagen fibres and were seldom encountered in isolation. In all the normal tissues the elastic fibres had a typical mature morphology (105).

Beneath this elastic layer lie the fat cells. The patients in the study had variable body mass indices and it was not possible to correct or correlate for this factor. The fat cells themselves were usually contained within the mesorectum and may occasionally project together with the elastic fibres into the sub peritoneal collagen layer. However, in the normal situation at no time were fat cells found to be in direct contact with peritoneum alone.

3.2. Structural effect of disease

3.2.1 Macroscopic effects

The most striking and obvious feature of advanced rectal prolapse is that the rectum protrudes externally. The extent to which this occurs is variable and in some cases less advanced protrusions which the patient can self-reduce are identified. Other patients may present with internal rectal prolapse, where the proximal rectum telescopes inside itself to then occlude the anus (see figure 12). More minor versions of these latter phenomena are recognised. However, these patients seldom require surgical correction and are not considered further in this thesis. The mucosal surface of the externally prolapsed rectum may be ulcerated and or bleeding; such advanced changes are not often seen with internal prolapse with the exception of solitary rectal ulcers which were not encountered in the study population.

From within the abdomen the appearances of a patient with prolapse are reasonably characteristic. The colon itself often appears elongated and floppy; in 40 rectopexies for prolapse the caecum was actually present in the pelvis compared with 25 cases where this was not so (see figure 28). A similar situation is also recognised with the sigmoid and transverse colon (although

not formally quantified here). In the prolapse itself the rectum often sits lower in the pelvis than in normal individuals (although this is difficult to quantify). In some cases there is recession of the pouch of Douglas with the associated development of an enterocele (figure 31). The peritoneal layer has a variety of appearances ranging from thickened to fat studded. Other small surrounding peritoneal defects are also recognised.

Table 19- Number out of 65 cases where a macroscopic intra-abdominal abnormality was present

Effect	Number of cases present
Pelvic caecum	40
Recession of pouch of Douglas	37
Enterocele	28
Peritoneal fat studding	18
Isolated peritoneal defects	22

Figure 28 Pelvic floor abnormalities affecting prolapse patients

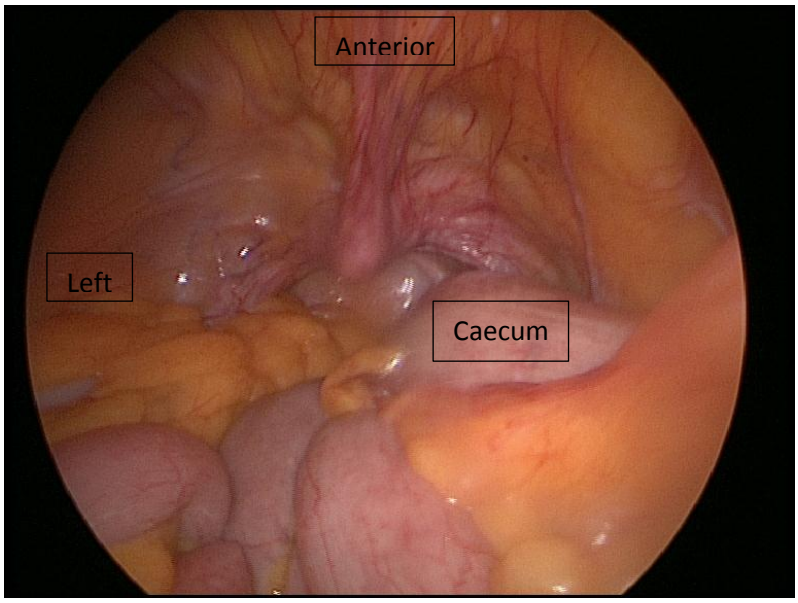
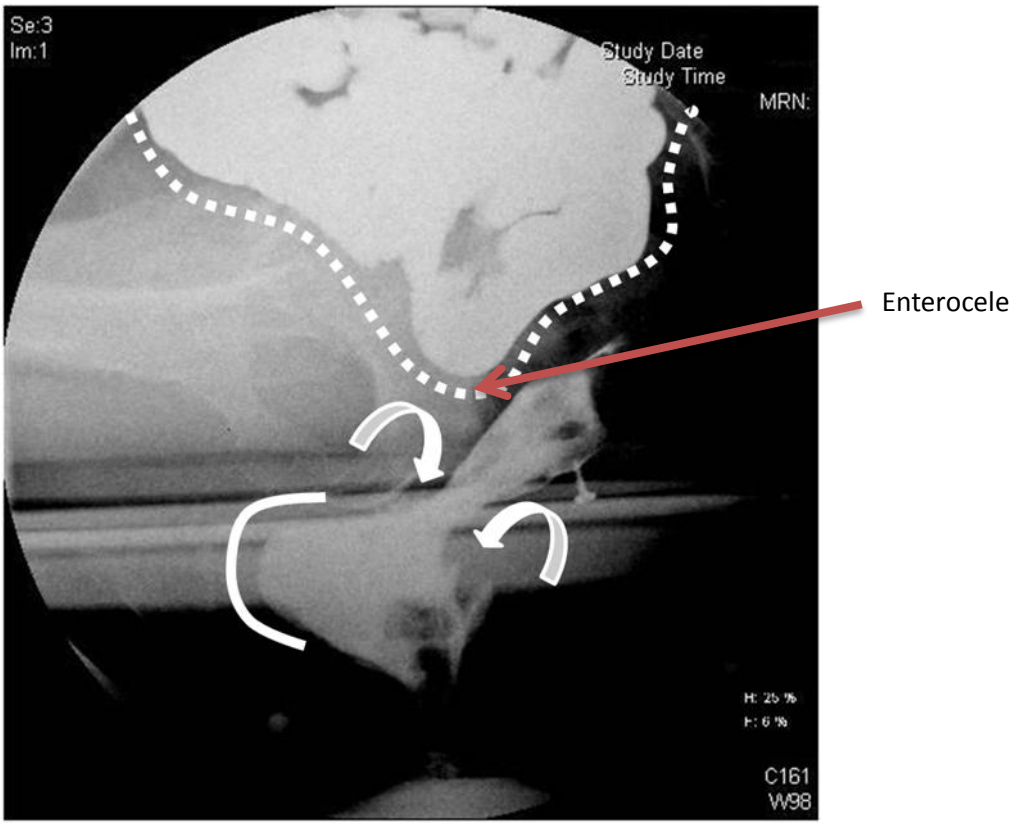


Image taken during laparoscopic ventral mesh rectopexy from a female with rectal prolapse. A urinary catheter has been inserted and the patient has previously undergone a hysterectomy. A large and highly mobile caecum is projecting deep into the pelvis beyond the sacral promontary, this is a highly consistent finding in patients with rectal prolapse.

Figure 29 Defecating proctogram



This is a radiograph taken from a middle aged female patient with symptoms of obstructed defecation. Oral contrast (containing barium) has been administered and fills the small bowel, located above the dotted line. The patient is sitting on a commode and barium containing contrast media has been inserted into the rectum. During attempted evacuation the various forces distorting the rectum are demonstrated. The most notable findings are those of a rectal intussceception (demonstrated by arrows), herniation of the rectum into the vagina (demonstrated by plain white line) and an enterocele (bowel protruding through pelvic tissues to compress rectum).

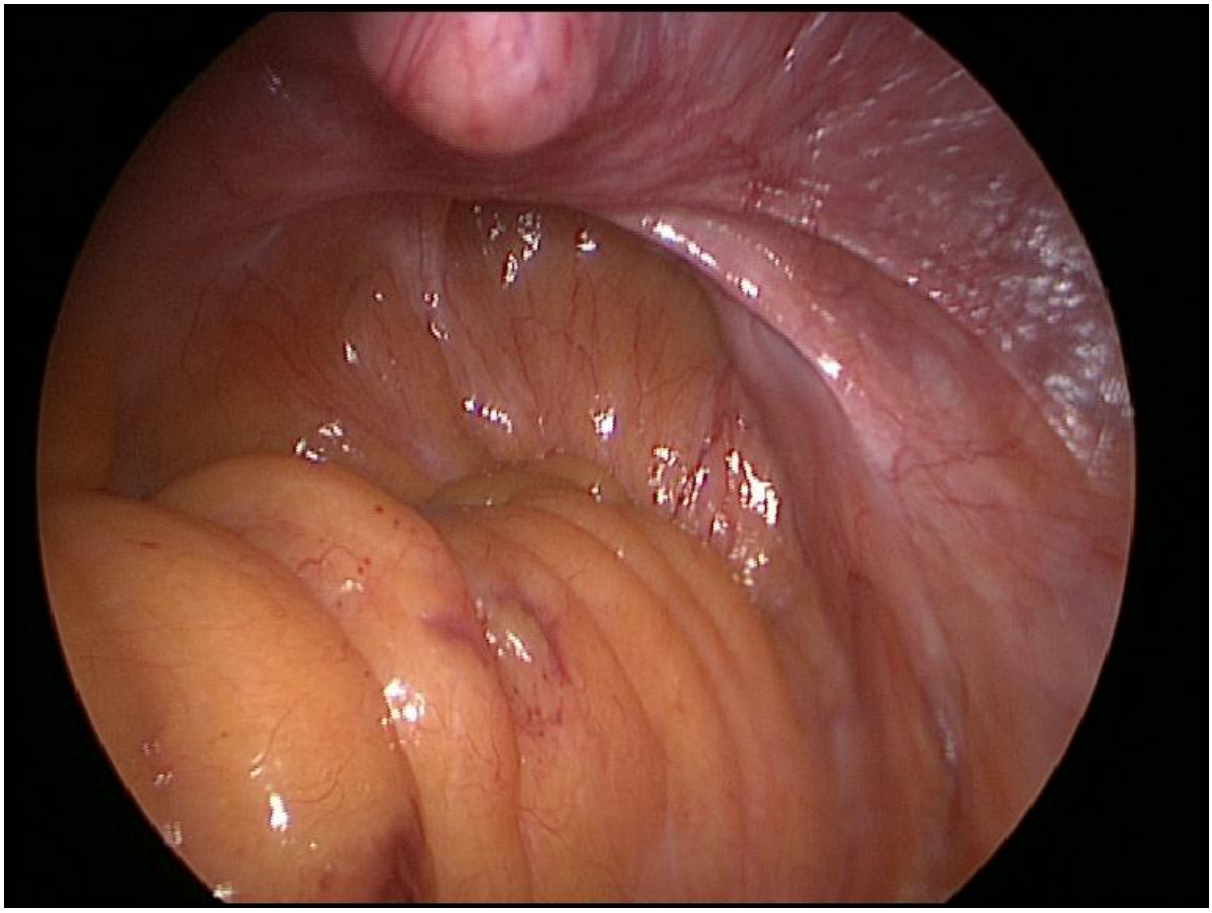


Figure 30 Pelvic floor changes in prolapse patients

Intra operative photograph of the pelvis in a multiparous women with external rectal prolapse undergoing a laparoscopic ventral mesh rectopexy. In this case the caecum was located entirely in the pelvis. The structures at the level of the peritoneal reflection are deeply recessed and mobile and the sigmoid mesocolon grossly thickened.

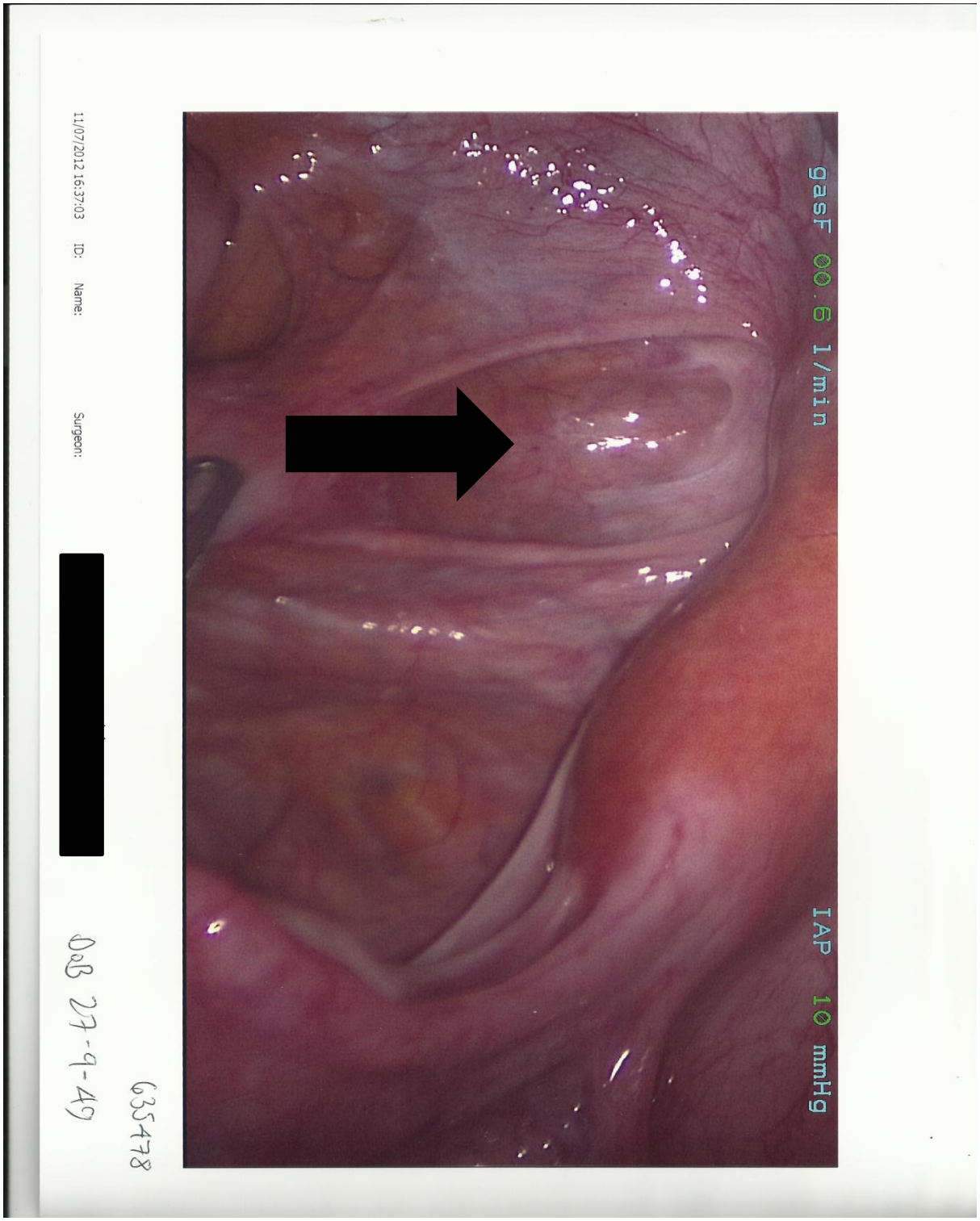


Figure 31 Structural failure of the Douglas Pouch

Laparoscopic view of the pelvic floor of a female with symptoms of internal rectal prolapse. The arrow demonstrates the deep recession of the Pouch of Douglas, a common finding.

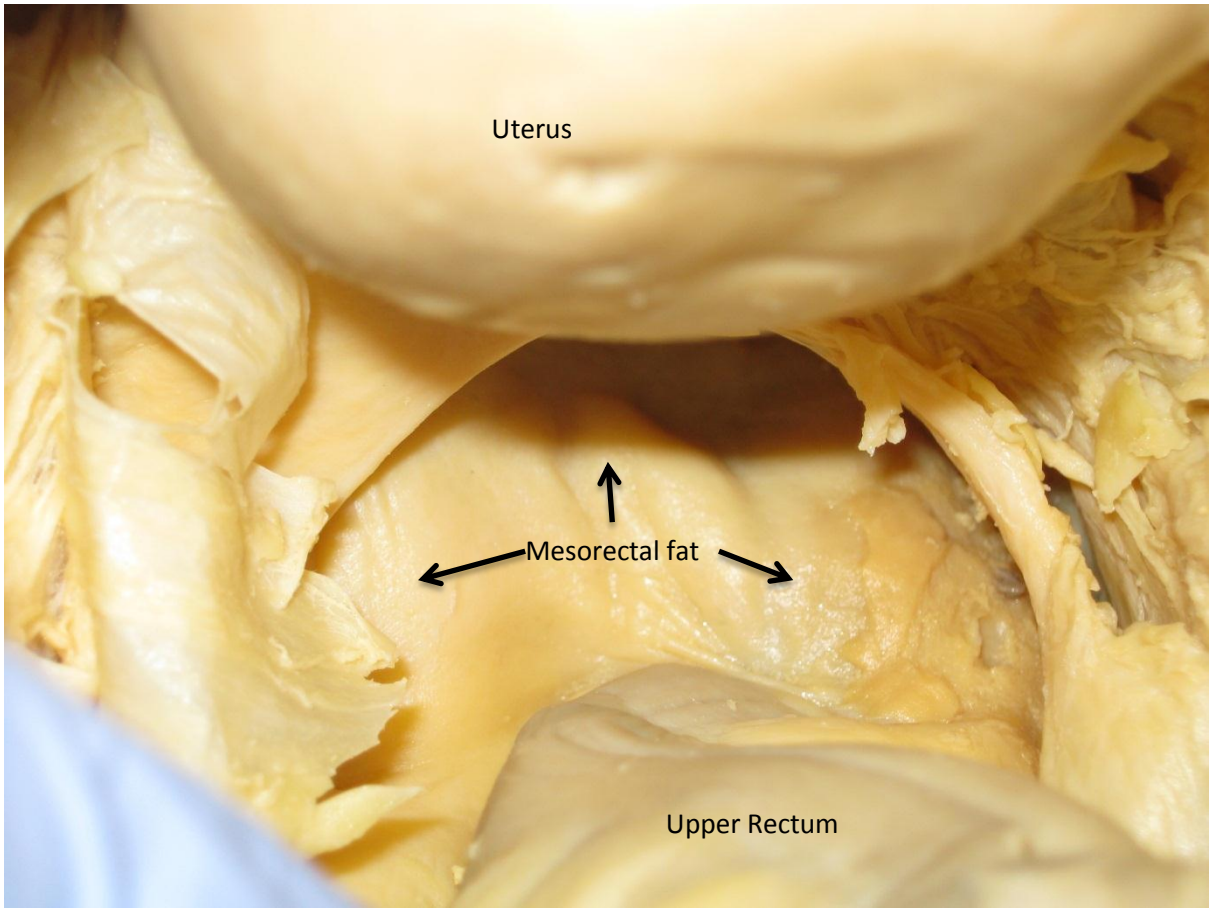


Figure 32- Normal view of the pelvic floor

Cadaveric dissection of female pelvis (the uterus is in situ). In this patient there is none of the stigmata of rectal prolapse present in image 28. The mesentery of the sigmoid colon is not thickened and the layer of the peritoneal reflection is not recessed as in the pathological case.

3.2.2 Microscopic effects of disease

Only the structural effects will be described here, all cellular responses are addressed in section 5.1. The ultrastructure of the sub peritoneal collagen layer is disrupted in the majority of patients with pelvic organ prolapse. This is most marked in multiparous women but was also seen in a proportion of men and nulliparous women with the condition. This is illustrated in table 20.

Table 20

Microscopic structural changes in pelvic connective tissues of patients with rectal prolapse.

Group	N	Collagen layer intact	Evidence of disruption	Elastic fibre disruption	Fat herniation
Control	5	5	0	0	0
Males	10	4	6	4	2
Nulliparous women	15	7	8	6	4
Multiparous women	45	16	29	24	23

The disruption to the collagen layer was often associated with defects in the elastic fibre layer.

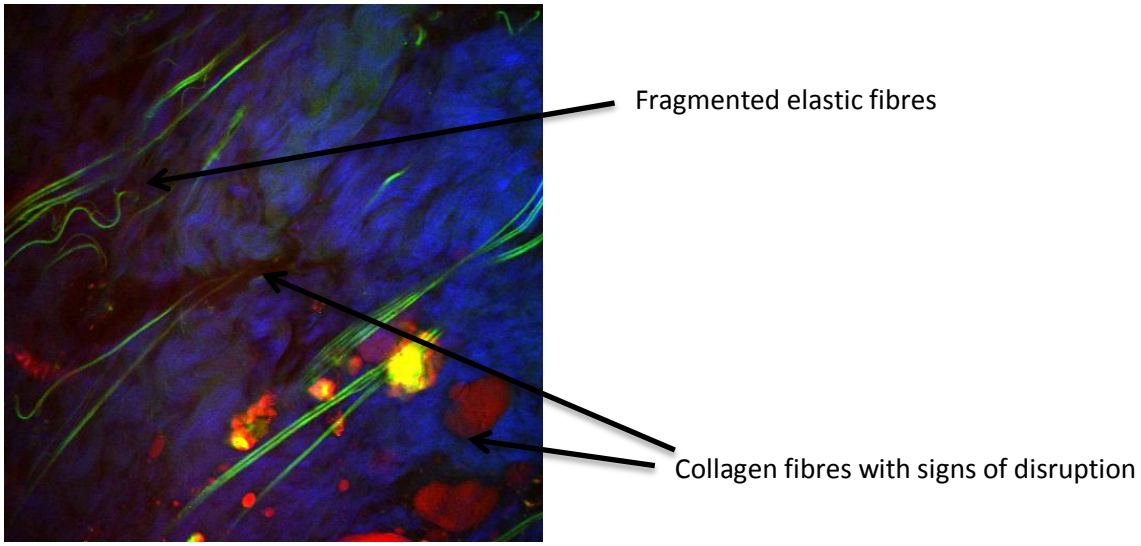


Figure 33 Disrupted pelvic connective tissues in rectal prolapse

The above image is taken from a male with rectal prolapse. The rectovesical pouch was dissected out and sagitally sectioned. The specimen was analysed using multiphoton light microscopy. Once the images were acquired these were digitally merged and colour added to facilitate the identification of the various structures (elastic fibres – green, collagen – blue and fat – red). In contrast to the control situation (figure 27) the disruption to the tissue structures can be seen, the elastic fibres are no longer organised into discrete layers and are found interspersed through the collagen layer in a random fashion. Because multiphoton microscopy involves minimal tissue dissection these effects are likely to represent the effects (or cause) of the prolapse process.

Attempts at repair and regeneration were evident in these tissues from prolapse patients, typically in the form of a thickened sub peritoneal collagen layers. Morphologically the connective tissues appeared very different from those observed in the control setting. In some cases the collagenous layer was completely deficient and therefore fat cells were in direct contact with the

peritoneal layer, a feature that correlates with the macroscopic appearances of these tissues and hernias at other bodily sites. In many cases the collagen identified at sites of injury was actually thicker than at uninvolved sites and controls. It was ,however, morphologically different.

The fascial bands were assessed by comparing controls with those patients suffering from obstructed defecation. In the control situation most participants were found to have thin fascial bands (89% of total) compared with those who had prolapse (66% of total). In the group with pathology there was an increase in the thickness of fascial bands, such that there were more thick fascial bands (11% vs. 23%) and a tendency for some patients to have no fascial bands at all (10%). These trends were not statistically significant (p>0.5 Chi Squared test 3 degrees of freedom). In addition no differences were noted in the fascial bands or sub peritoneal collagen thicknesses in patients with different stages of the disease (p =0.25 Chi Squared test 4 degrees of freedom).

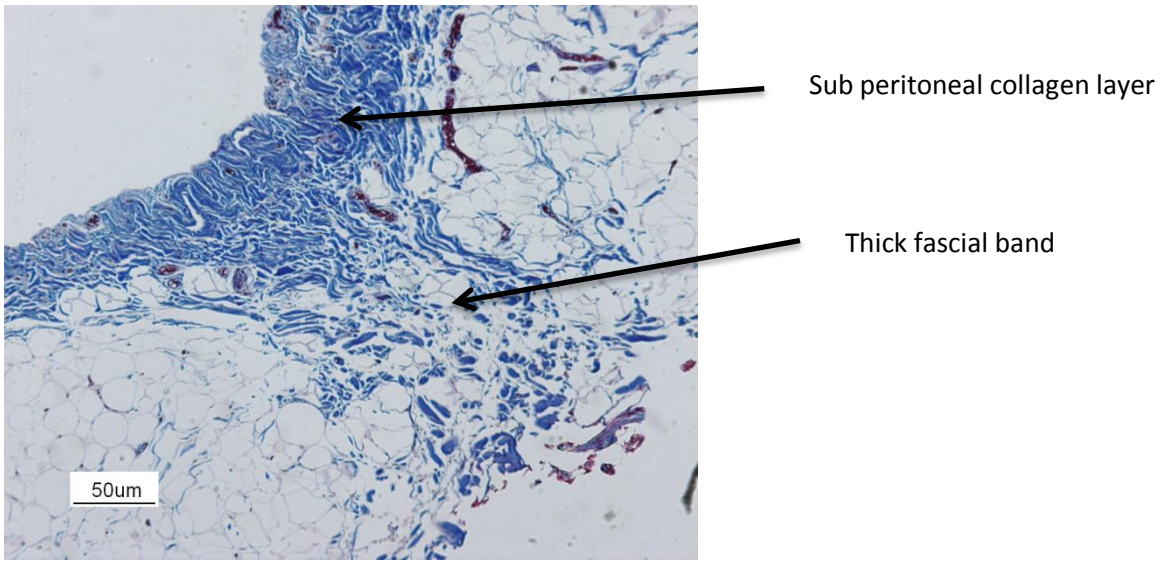


Figure 34 Fascial bands supporting the rectum

Histological section of the pelvic connective tissues of a patient with rectal prolapse stained using Masson Trichrome (Collagen – blue) to demonstrate a thick fascial band traversing the mesorectal fat.

Chapter 4.0 Effect of joint hypermobility on outcome following surgical procedures for rectal prolapse

Patients with rectal prolapse are a highly heterogeneous population. Our previous casual observations demonstrated that there were several dominant groups. The largest group comprises elderly multiparous females who are post menopausal. The other groups include younger women, some of whom have only a minor obstetric history or may even be nulliparous. Males present at a variety of ages and cannot be classified according to parity or menopausal status.

4.1Effect of joint hypermobility on characteristics of patients undergoing surgical treatment for rectal prolapse and recruited for this study; age at presentation and history of previous surgical treatments

Previous authors had noted that individuals with benign joint hypermobility syndrome had a high incidence of unexplained gastrointestinal symptoms (115).

We aimed to study this situation in greater detail. We prospectively recruited patients and evaluated their connective tissue status as described in section 2.1. These patients therefore comprised two groups, those with normal connective tissue status and those who had benign joint hypermobility syndrome.

A total of 52 patients were assessed. Of these, 18 fulfilled the diagnostic criteria for benign joint hypermobility syndrome. Their results are summarised in table 21:

Table 21

Illustration of patient characteristics and prior interventions in patients with BJHS vs. Normal connective tissues.

Group	N	Median age	Age range	Internal prolapse	External prolapse	Number of procedures
Benign joint hypermobility syndrome	18	52	25-79	15	3	14
Normal	34	61	22-84	28	6	10

Patients with benign joint hypermobility syndrome typically present at a younger age than those without the condition. As a result the median age at presentation in those with BJHS was usually

at the pre or peri menopausal age of 51. In contrast those who did not have BJHS tended to present later, at the more typical post menopausal age of 61. This represents a 9 year difference between the two groups, a significant finding (T-Test $p<0.01$). In reality those who had BJHS may have actually developed the disease at an even younger age, this is because we counted the age at presentation for definitive surgery in our unit, rather than age at first intervention. The individuals who had BJHS were far more likely to have undergone previous surgical intervention for treatment of rectal prolapse or a disorder directly related to it than those who did not have the condition. Overall 83% of those with BJHS had undergone such interventions, contrasting with only 32% of those without BJHS who had undergone interventions. This too was a significant finding (Chi Squared test $p<0.01$). We further hypothesised that the disease extent in these younger patients would be greater than those without BJHS. However, the difference in the extent of the disease between the two groups was not significant (Chi Squared p 0.97).

4.2 Effect of joint hypermobility on patient re-operation rates following surgery for rectal prolapse

We sought to determine whether pre-existing systemic connective tissue disorders had an impact on surgical outcomes following laparoscopic ventral mesh rectopexy. Participants (that are described in table 21) were followed up over a 1 year period following their initial surgery. All surgical re-interventions for each patient were documented.

A total of 42 patients were analysed. Of these, 16 fulfilled the Beighton Criteria for benign joint hypermobility syndrome. Their results are summarised in table 22:

Table 22

Comparison of re-intervention rates post laparoscopic ventral mesh rectopexy in patients with BJHS vs. Normal connective tissues.

Group	N	Median age	Age range	Number of re-interventions
Benign joint hypermobility syndrome	16	50	25-78	5
Normal	26	58	23-84	2

Patients with benign joint hypermobility syndrome had a re-intervention rate of 31%, compared to 7% in those who did not have the condition (Chi Squared test p=0.03). Not included in this data were those patients who required behavioural (non operative) interventions post operatively which was required in 6% of those with benign joint hypermobility syndrome and in 19% of those who did not have the condition.

Chapter 5

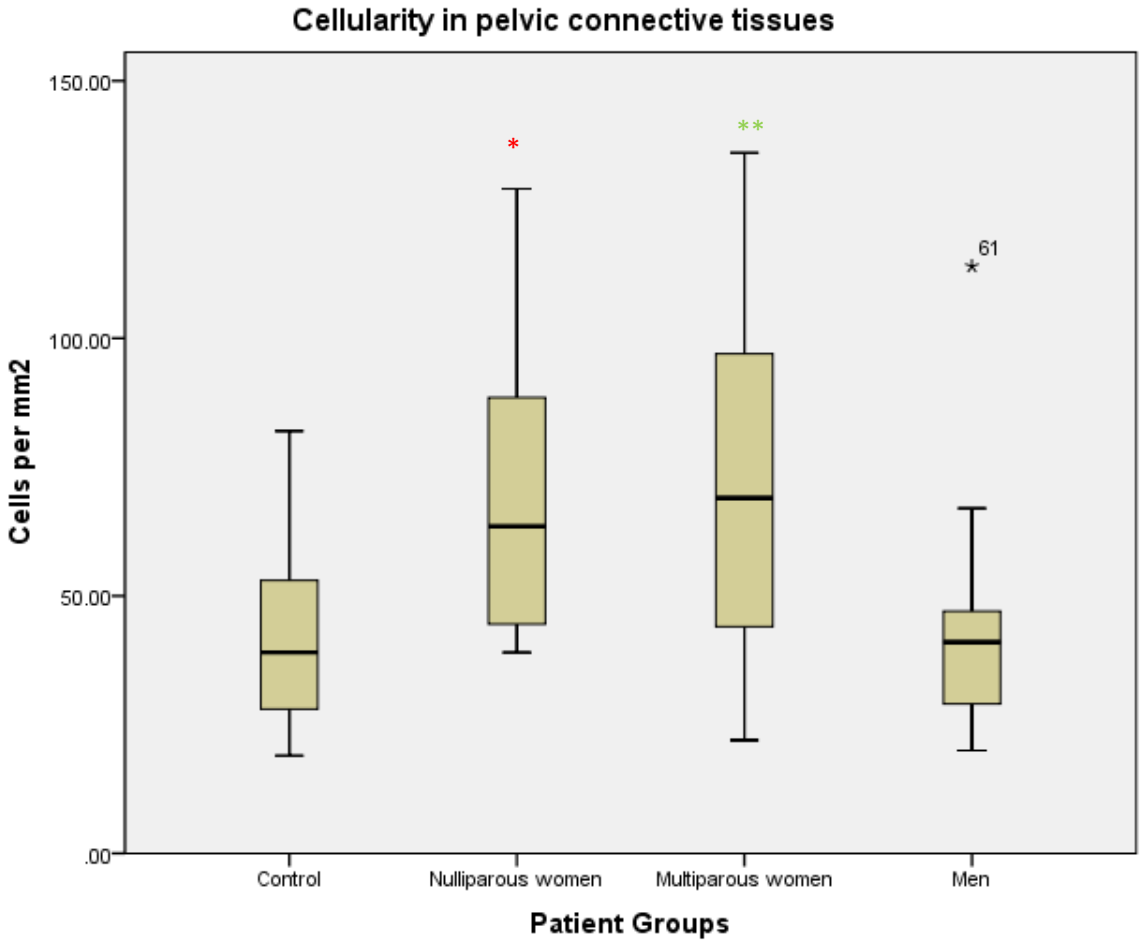
5.1 Cellularity studies in pelvic connective tissues

The pelvic connective tissues are populated with cells located within the extra cellular matrix. In the control situation the cells present in the extra cellular matrix comprised a quiescent population of fibroblasts. In view of the connective tissue trauma occurring in the pelvic connective tissues of those with rectal prolapse we hypothesised that the cellular population of the pelvic connective tissues in these patients would change. We studied this process by taking pelvic connective tissue samples from those individuals with prolapse and making comparisons with controls. Patients examined included 6 controls, 12 nulliparous women, 9 men and 38 multiparous women. Samples were processed and stained using haematoyllin and eosin and then viewed to identify regions of maximal cellularity from which absolute counts per high power field were taken (as described in section 2.8.1). Individuals who had rectal prolapse were found to have an increase in cellularity compared to controls.

The cellularity of the pelvic connective tissues in the patient groups is demonstrated in table 23 and graph 1 below;

Table 23 Cell counts per high power field in pelvic connective tissues of patients with rectal prolapse Vs control

Group	N	Mean	Median	Range	Standard Deviation
Control	6	43	39	19-82	22.3
Nulliparous women	12	70	64	39-129	32.5
Men	9	46	41	20-114	28.9
Multiparous women	38	71.2	69	22-136	29.5



Graph 1 Cellularity of pelvic connective tissues in different groups with prolapse.

This graph illustrates the cellularity of the pelvic connective tissues in patients with rectal prolapse compared to controls. Patients were categorised according to parity and sex. It was found that males had decreased cellularity of the pelvic connective tissues compared to females. Data was analysed using Students T- Test * p=0.06, **p=0.03.

The cells which increased were typically fibroblasts (described in section 1.8.2) and these were located in the collagenous bands of the pelvic tissues. The multiparous women demonstrated the greatest increase in cellularity compared with control and the male subjects the least; this was mirrored by the statistical findings which failed to show a significant result in the male group (p=0.08) and borderline results in nulliparous women (p=0.06).

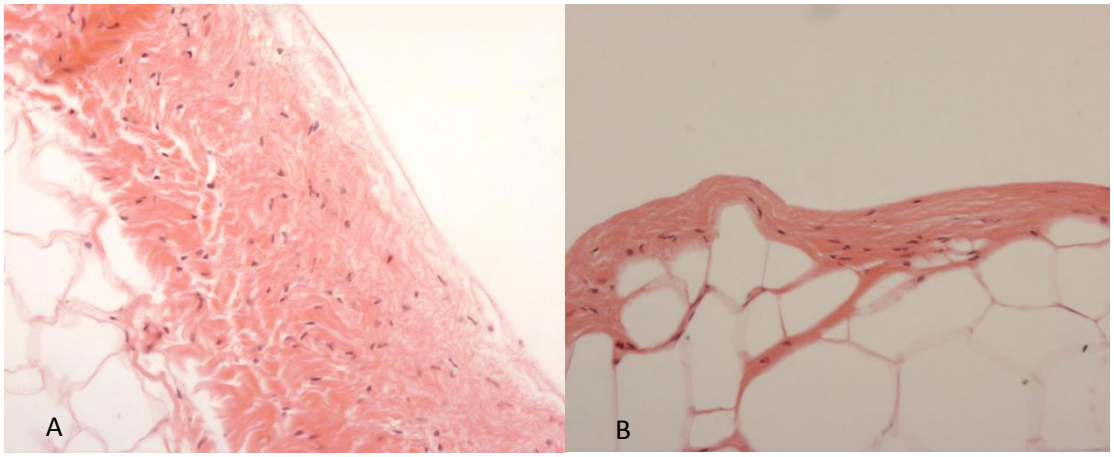


Figure 35 Cellularity of pelvic connective tissues of prolapse patients

These two images are taken from the pelvic connective tissues of a multiparous female with rectal prolapse (A) and a male with rectal prolapse (B). The tissues were sectioned in the sagittal plane and histologically stained using haematoxylin and eosin and viewed at 100x magnification with a light microscope. These two images illustrate the marked differences in cellularity between male and female pelvic connective tissues. These two sections also highlight the difference in thickness of the collagenous layer that was sometimes noted between male and female patients. In many cases men had fewer cells because they had less collagen (and it was in this layer that cellularity was greatest).

We suspected that there may be a difference in this cellular response within patients suffering from the more advanced form of the disease, because it would seem that these have the greatest forces imparted on the tissues. However, comparison of patients according to disease severity showed a slight trend towards increased cellularity in those suffering from external prolapse (table 24). This trend towards an increase was not statistically significant.

Table 24

Comparison of cellularity according to extent of prolapse. Results analysed using T Test (p=0.35).

Group	N	Mean	Median	Range	Standard Deviation
Internal prolapse	37	68	58	20-136	32.8
External prolapse	14	77	74	25-129	30.5

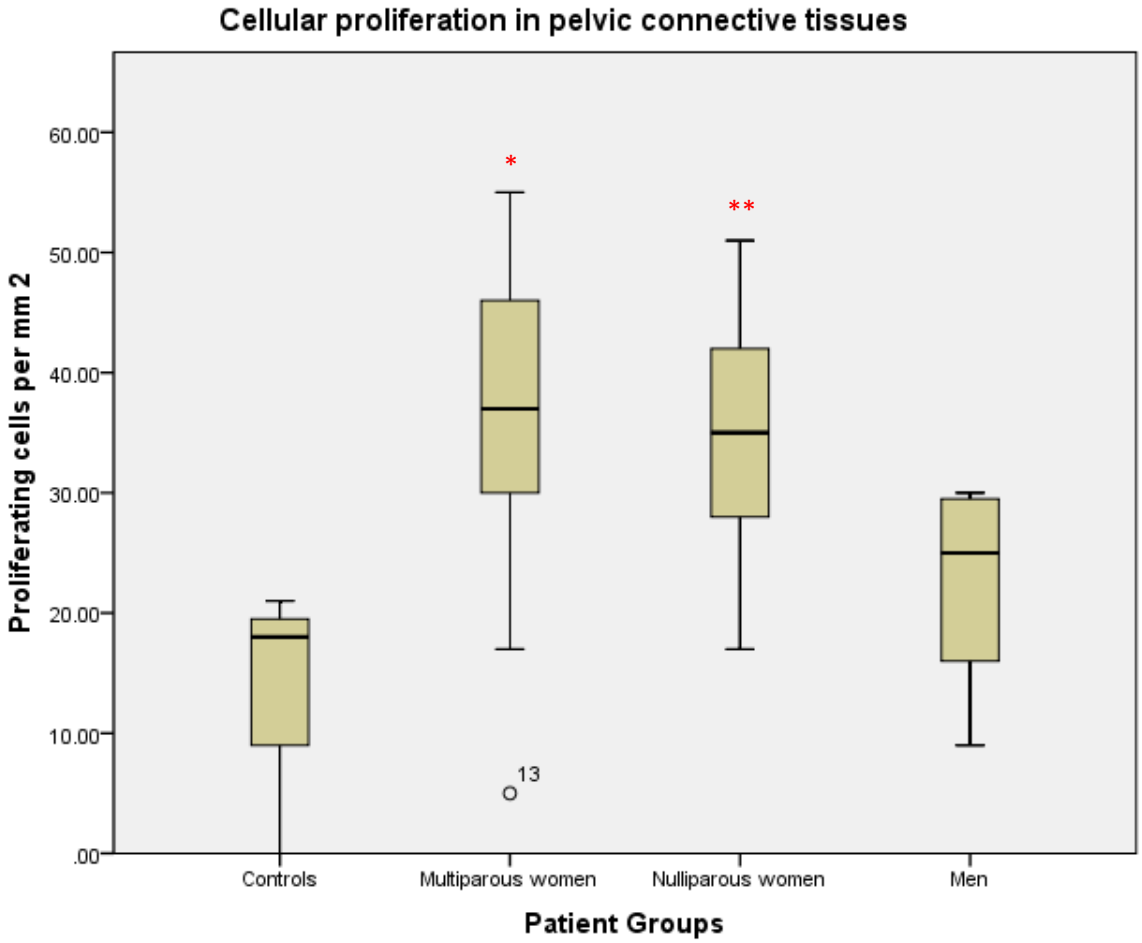
5.2 Cellular proliferation in pelvic connective tissues in response to prolapse

The increased cellularity identified in the pelvic connective tissues may occur via a number of processes. Cells may migrate to the tissues from elsewhere and then differentiate, or the existing cellular population may proliferate. To address this question we studied the cellular proliferation in the pelvic connective tissues using a cellular proliferation marker antibody (section 2.10.2). This stained those cells that were undergoing mitosis, but not those cells that are in the dormant stage of the cell cycle. Our results showed that the fibroblast population appears to increase by proliferation of the resident cellular population rather than by migration from other tissue areas. The proliferative activity was most marked within the sub peritoneal collagen layer. Additional proliferative activity was also identified in the fibroblasts populating the pararectal fascial bands. The data for cellularity is given in the table 25 and graph 2.

Table 25 Fibroblast proliferative activity in pelvic connective tissues of patient groups

Group	N	Mean	Median	Range	Standard deviation
Control	4	13	18	0-21	11.3
Nulliparous women	9	35	35	17-51	12
Males	7	22	25	9-30	8.9
Multiparous women	17	35	37	5-55	12.6

Comparison of fibroblast proliferative activity (as assessed using antibody to Ki67) between the patient groups.



Graph 2 Cellular proliferation in pelvic connective tissues of patients with rectal prolapse

In this graph the cellular proliferative activity across the patient groups is demonstrated. Samples were stained using Ki67 antibody to identify cellular division. The greatest proliferative activity was found in multiparous women. Results were analysed using Students T – Test (sample vs. control) *p=0.05, **p=0.05, the result for males was not significant p=0.38.

In common with the findings that male connective tissues had reduced cellularity. It was noted that the cells in these patients did not seem to proliferate in response to prolapse to the extent noted in multiparous women. In the control situation it is evident that some proliferation of resident cells occurs but is less than in response to the disease process of prolapse.

5.3 Cellular differentiation of cells in pelvic connective tissues

The fibroblasts in the pelvic connective tissues may be differentiated to sub-serve a specific function, or remain undifferentiated. In the context of this work we sought to determine whether

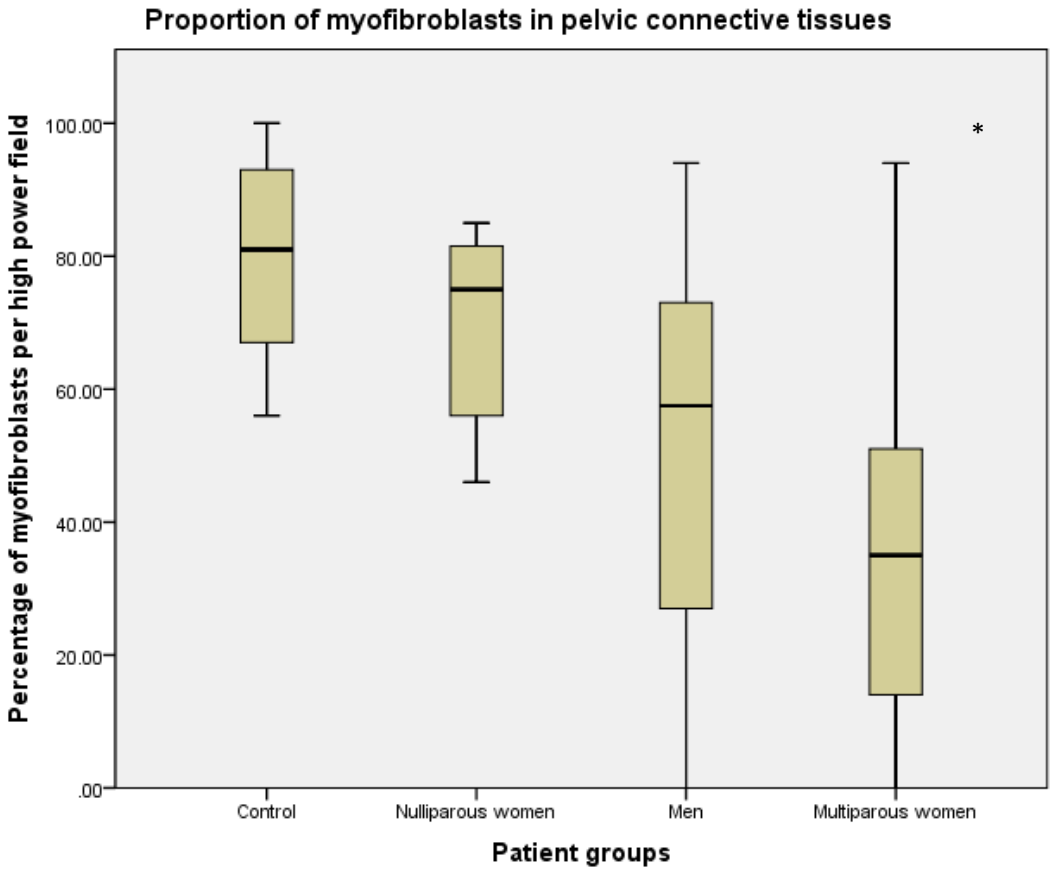
they had developed a myofibroblast phenotype. This confers upon fibroblasts a series of contractile properties. They are most evident, and perhaps most important, during the latter stages of human wound healing (102) (for further details see section 1.8.3). This was studied by taking the pelvic connective tissues and undertaking immunohistochemistry staining to determine whether the fibroblasts had developed actin filaments which are seen in myofibroblasts(107). In the control situation the pelvic connective tissues were found to contain myofibroblasts, and these constituted the predominant type of cell. In the disease situation the males also demonstrated this cell type, though the amounts were lower than in the control situation. In multiparous women, who exhibited some of the greatest increases in cellularity, the proportion of myofibroblasts was lower. The myofibroblast results are summarised in table 26 and graph 3

Table 26- Myofibroblasts in pelvic connective tissues

Results expressed as percentages (myofibroblasts/ total cell number).

Group	N	Mean	Median	Range	Standard deviation
Control	6	79.7	81	56-100	16.3
Nulliparous women	7	68.7	75	46-85	15.7
Males	10	50	57.5	0-94	32
Multiparous women	21	38	38.5	0-100	30.1

In this table the proportion of myofibroblasts in the pelvic connective tissues of the patient groups vs control are demonstrated. It was found that myofibroblasts constituted the dominant cellular subtype in the pelvic connective tissues of controls. In those suffering from rectal prolapse the proportion of myofibroblasts was decreased, with the exception of nulliparous women.



Graph 3 Myofibroblast proportions in pelvic connective tissues

Graph showing the myofibroblasts in the pelvic connective tissues of patients Vs controls. Results were analysed using students T test (Sample vs. Control). *p=<0.001. The results for nulliparous women and men were not significant.

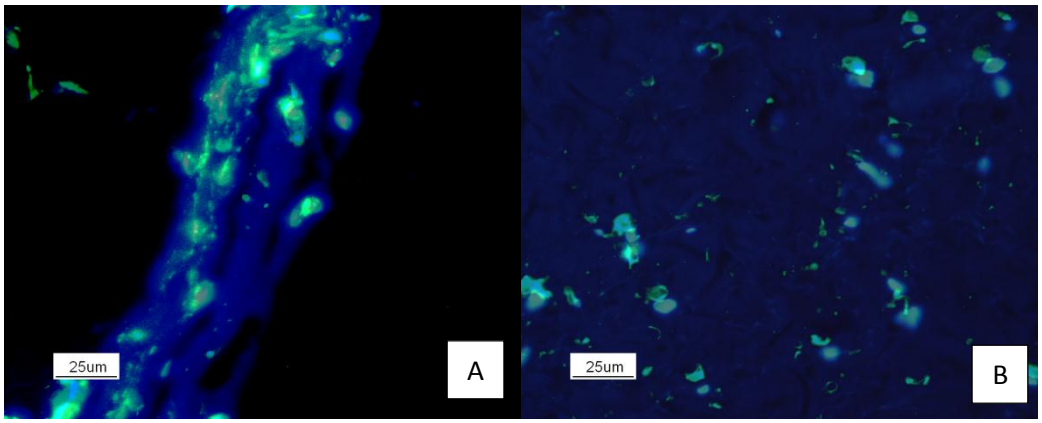


Figure 36 Myofibroblasts in pelvic connective tissues

Samples of pelvic connective tissues from controls (A) and multiparous female with rectal prolapse (B). Sections are stained to demonstrate myofibroblasts (green). In the control sample the large proportion of myofibroblasts relative to normal cells (blue) can be seen. In sample B evidence of myofibroblasts can be seen, however there are fewer than in the control setting.

The data above support the idea that in nulliparous women the increased cellularity of pelvic connective tissues that occurs in nulliparous women is accompanied by a process of cellular differentiation. In males the proportion of myofibroblasts is lower suggesting that they have either de-differentiated or that they are replaced by a less specialised cellular subtype.

5.4 Cell studies summary

The data presented supports the idea that the resident cell population in the pelvic connective tissues of patients with rectal prolapse alters in response to the disease process. In most groups the cellular density of the pelvic connective tissues increases and this process occurs via the proliferation of the resident cell population. The phenotype of this cellular population alters with respect to the myofibroblasts that are present, with multiparous females showing the lowest proportion of this cell type.

Chapter 6-

6.0 Matrix metalloproteinases

Cells of the extracellular matrix (ECM) produce both matrix macromolecules and agents involved in tissue turnover. The stability of the matrix depends on the balance between these two processes. If rates of biosynthesis and degradation of matrix macromolecules are in balance, the tissue composition remains stable. However if rates of production and activation of proteases which breakdown matrix increases relative to rates of biosynthesis, the tissue tends to degrade

The tissues from prolapse patients and controls were thus investigated for presence of a number of relevant proteases and also tissue specific inhibitors potentially involved in turnover of pelvic connective tissues. Details of the immunohistochemical methods used are given in Section 2.7 and 2.9 , details of the antibodies used in table 10

6.1 Matrix metalloproteinase 1.

Here we studied MMP 1 initially; this is a collagenase capable of cleaving mature collagen fibrils(135). In order to assess this process immunohistochemistry was undertaken using antibodies targeted to MMP1. This allows accurate localisation of the protease and an estimation of the amount of enzyme present, through histological grading of positive cells and tissues (as described in section 2.11).

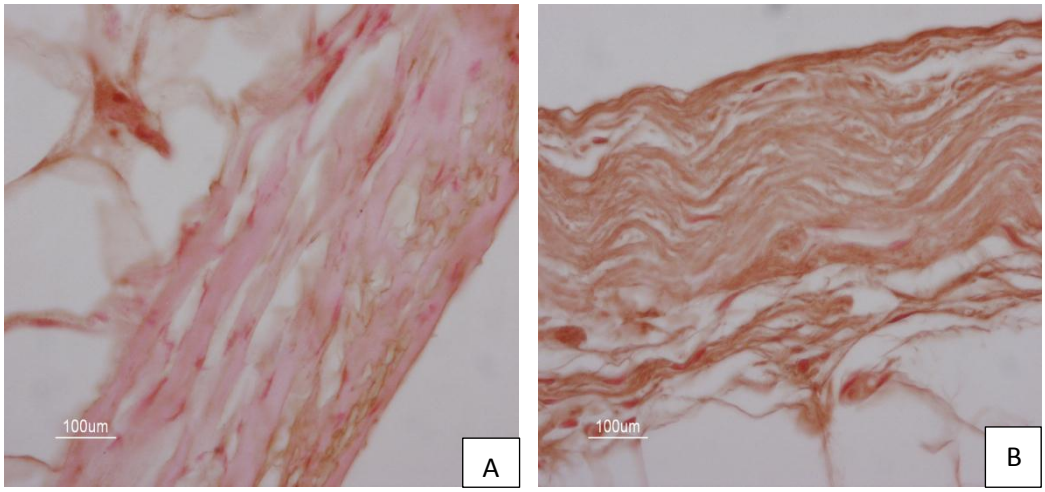
In the pelvic connective tissues of patients with prolapse the most intense staining for MMP 1 was noted in multiparous females. Males tended to show slightly lower levels of MMP 1 expression with 43% having grade 3 MMP 1 expression. Within the male group 14% showed grade 1 MMP 1 staining which was the situation in 75% of the control group. MMP 1 was largely located within the extracellular matrix, and was most concentrated around the collagen in the sub peritoneal collagen layer and fascial bands. Due the nature of the microwave digestion process it was not really possible to make accurate assessments of the structural integrity of

these areas on the immunohistochemically stained tissue. However, this was undertaken on tissues stained via alternative methods.

Table 27 – MMP 1 Staining in pelvic connective tissues of patients with prolapse Vs controls

Group	Grade 0 (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
Control	0	100	0	0
Men	0	14	43	43
Multiparous women	0	12	18	70
Nulliparous	0	0	60	40

Figure 37- Tissue staining for MMP 1in the pelvic connective tissues of patients with rectal prolapse



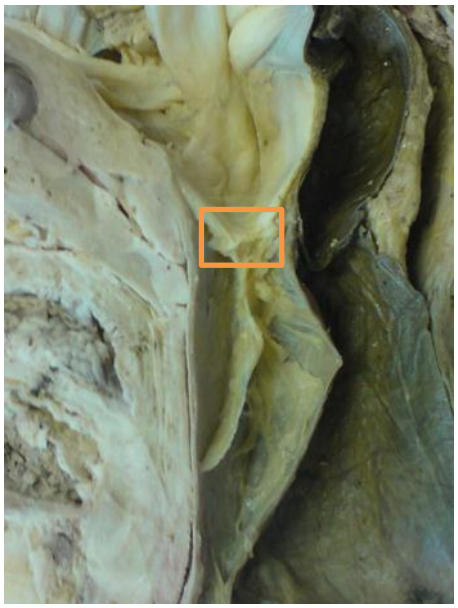


Image A is taken from a control sample (see orientation diagram above for specimen origin). This demonstrates very little MMP1 staining. Image B is taken from a male with rectal prolapse and demonstrates strongly positive staining for MMP 1.

The increased proteolytic activity that is noted in some patients may result in an overall decrease in connective tissue integrity. To investigate this further the connective tissue strands travsersing the mesorectum were utilised as a proxy marker. We have noted that these connective tissue bands are present in the normal situation and in some patients with prolapse. We aimed to determine whether the increase in MMP 1 staining was associated with any changes in the fascial bands running through the mesorectal fatty layer.

When MMP1 staining intensity was compared with fascial band thickness it was found that MMP1 grade 3 staining was associated with a reduction in the thickness of fascial bands and in 25% cases these were entirely absent (a phenomena which has been observed operatively but is difficult to quantify). This is contrasted by the results obtained when grade 2 MMP1 staining is compared and in this group thick fascial bands were seen in all patients with prolapse. The work on studying fascial bands was conducted on the multiparous females (with internal prolapse) to attempt to minimise any confounding effects posed by a potential underlying tissue disorder.

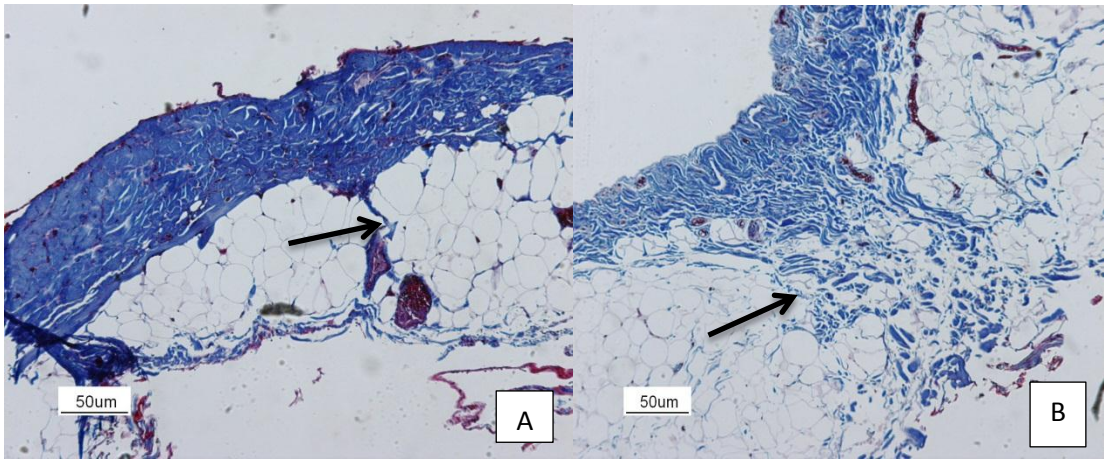
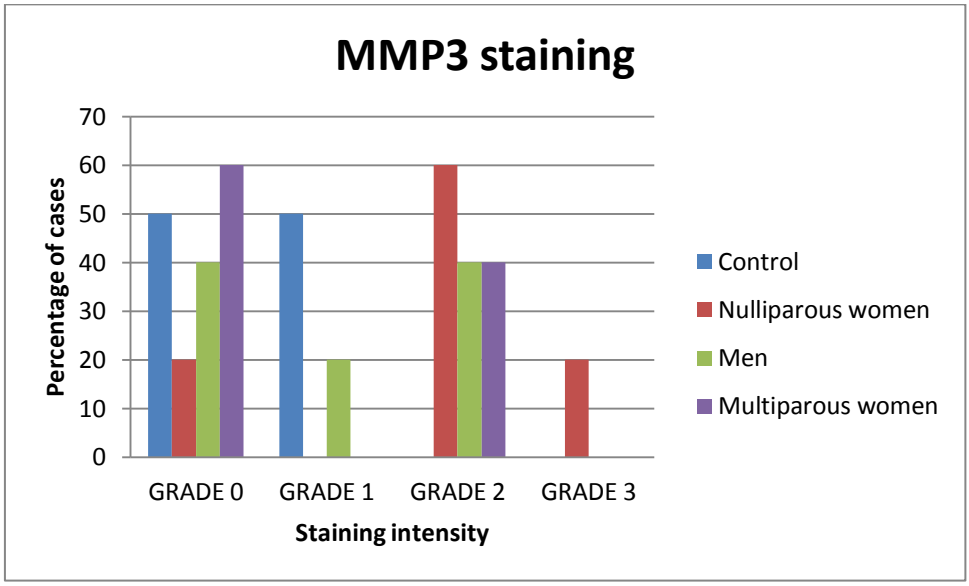


Figure 38- Comparison of fascial band thickness in pararectal connective tissues.

These two images are taken from sections of pelvic connective tissues from multiparous females with rectal prolapse, they have been stained using the Masson Trichrome method. In these two pictures the fascial bands are more clearly demonstrated. In sample A, a solitary scanty fascial band is seen (arrow), in sample B a much thicker structure is apparent (arrow).

6.2 Matrix metalloproteinase 3

MMP3 is another member of the class of matrix degrading enzymes, it is unable to degrade fibrillar collagen, but is able to degrade other components of the ECM, as well as activating other matrix enzymes. We investigated MMP3 activity in the pelvic connective tissues of patients and controls. In control tissue MMP 3 was either absent or its presence indicated by grade 1 staining (50%). In patients with prolapse there was stronger staining of MMP 3 indicating increased levels in some individuals. This is displayed in graph 4.



Graph 4 MMP 3 staining in pelvic connective tissues of patients with prolapse

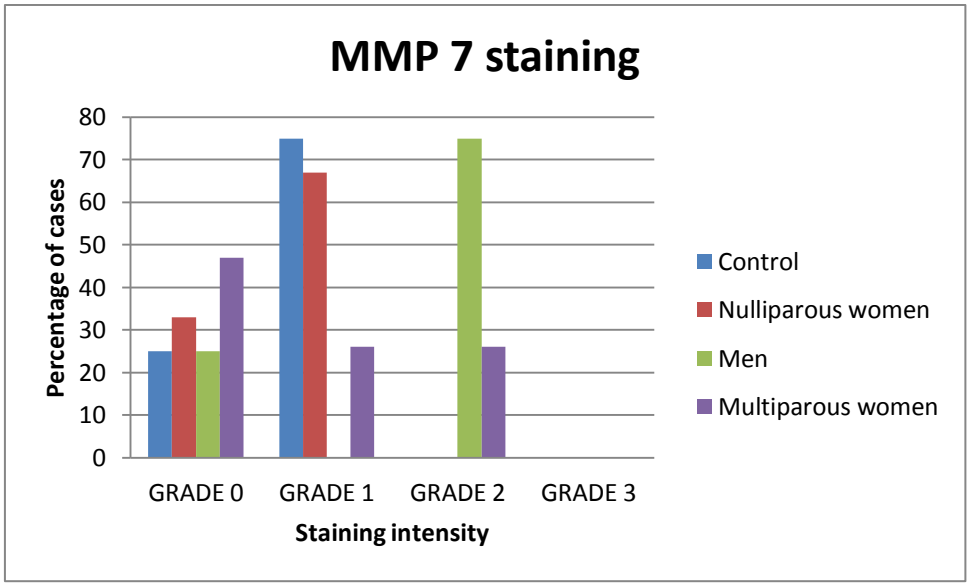
MMP 3 activity was studied in the pelvic connective tissues of patients with rectal prolapse Vs. controls. Increased staining was only seen in association with rectal prolapse and nulliparous women showed some of the strongest staining, indicating an increase in MMP3 in the connective tissues of this group.

The multiparous women and men did not show the marked increases in MMP3 activity that were noted in nulliparous women, this may indicate a decreased tendency for degradation of the other components of the ECM in this group.

6.4 Matrix metalloproteinase 7

MMP7 is one of the matrix enzymes that is not capable of cleaving fibrillar collagen, it is capable of activating MMP3 and MMP9. We investigated MMP7 activity using immunohistochemistry and

targeted antibodies to MMP7. Patients were sub-grouped according to gender and parity. Most cases did not show a marked tissue staining for MMP 7. Some individuals had strongly positive cellular staining for MMP 7 perhaps indicating that is was synthesised but had yet to be released. A small group of the multiparous women and the majority of the men did show increased staining for MMP 7 (graph 5).



Graph 5 MMP 7 Staining in pelvic connective tissues of patients with prolapse

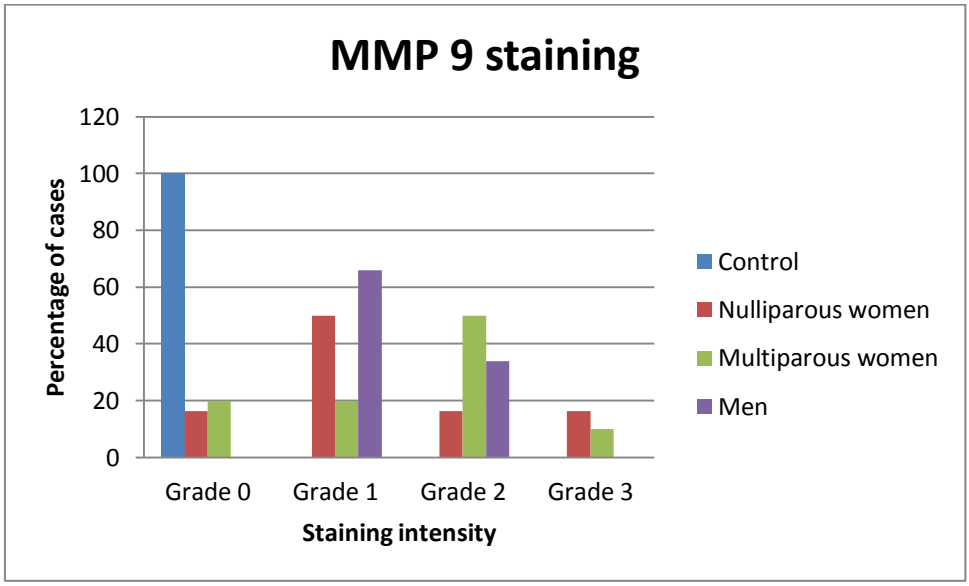
The concentration of MMP 7 in the pelvic connective tissues of patients with prolapse Vs controls is compared. It was found that males and some multiparous females show the greatest increases in MMP7 activity. On the basis of the experiments performed the biological significance of this is unclear.

In no cases was strongly positive tissue staining for MMP 7 identified. In cases where MMP 7 was identified it was localised to the sub peritoneal collagenous connective tissue layer and to a lesser extent the fascial bands traversing the mesorectum.

6.5 Matrix metalloproteinase 9

MMP 9 is another matrix degrading enzymes , that has previously been reported to be capable of degrading elastic proteins. We therefore investigated this enzyme and its concentration using immunohistochemistry with antibodies targeted to MMP9. We performed this analysis on the

pelvic connective tissues with prolapse. We did not conduct the experiments of the dermal samples as these did not show any evidence of dynamic tissue remodelling. Increased staining for MMP 9 was noted in the pelvic connective tissues of all patient groups. A small proportion demonstrated no positive staining. In most cases positivity was confined to the sub peritoneal collagen layer and the fascial bands. In the control tissues there was no MMP 9 positive staining identified. The proportions are illustrated in graph 6:



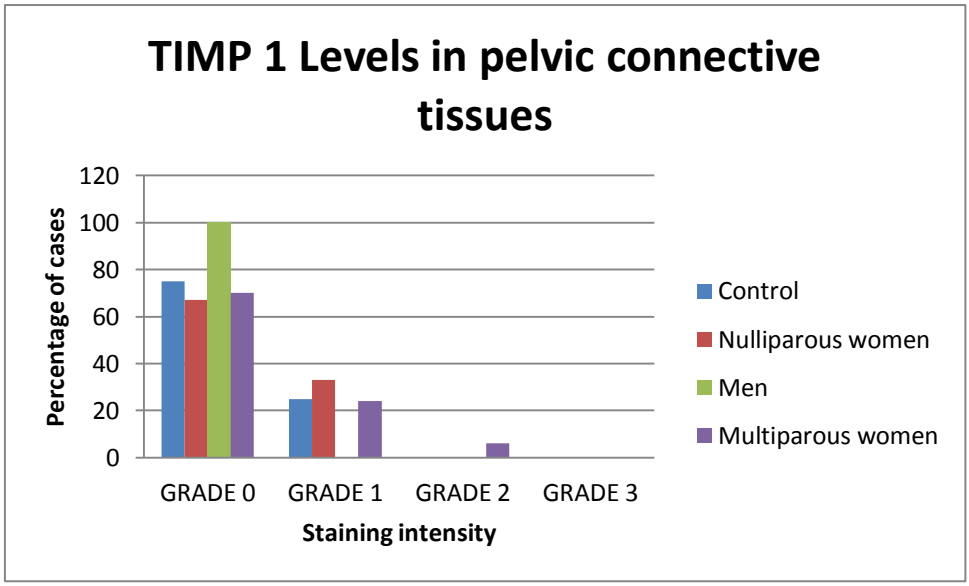
Graph 6 MMP 9 Staining in pelvic connective tissues of patients with prolapse.

MMP 9 activity was studied in the pelvic connective tissue of patients with prolapse compared to controls. In the control situation no MMP 9 activity was demonstrated. In those patients suffering from rectal prolapse increased MMP 9 activity was seen in most cases.

6.6 Tissue inhibitor of matrix metalloproteinase 1

Matrix degrading enzymes are associated with an endogenous inhibitor; in the case of TIMP1 it is capable of inactivating a range of matrix enzymes and MMP1 in particular. In order to determine whether the increases in MMP1 that were noted in the pelvic connective tissues of prolapse patients were likely to represent biological activity, TIMP1 was studied. Patients were grouped according to parity and gender. Analysis was conducted using immunohistochemistry with antibodies targeted to TIMP1 (table 10).

In the pelvic connective tissues there was very little TIMP 1 either in controls or in those with prolapse (graph 7).



Graph 7- TIMP 1 in pelvic connective tissues

TIMP 1 activity was assessed in the pelvic connective tissues of patients with prolapse compared with controls. In all groups very little TIMP 1 was present, suggesting that the MMP increases that were seen in some patients may be capable of biological activity.

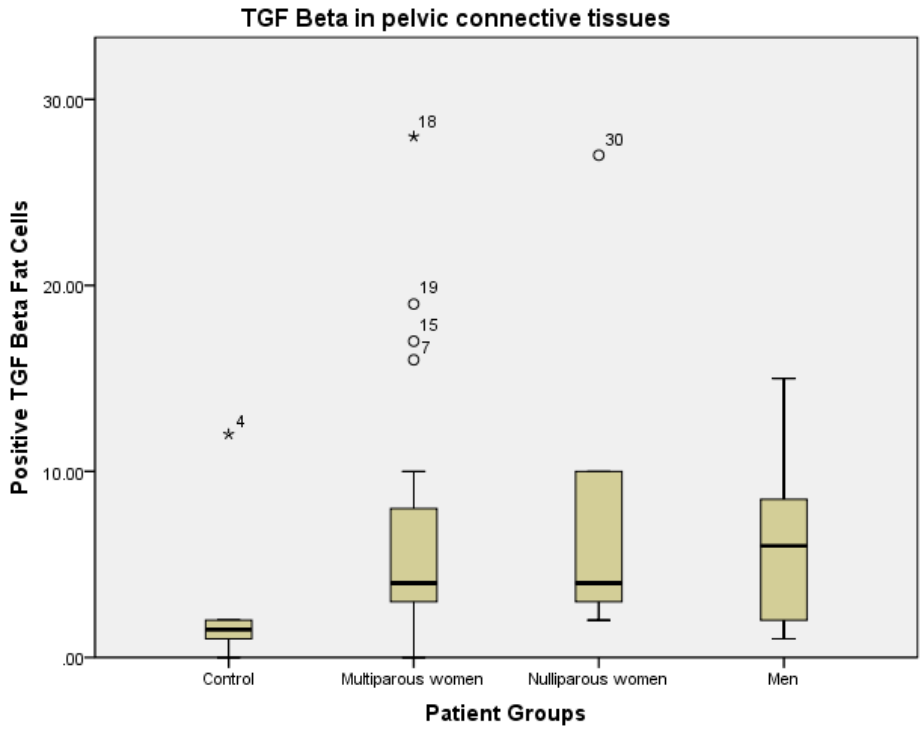
A small number of multiparous women (who had internal prolapse) did have increased levels of TIMP 1. None of the men showed any increases whatsoever. A cluster of patients also showed grade 1 staining, all had internal prolapse only. A small number of the controls also had increases in TIMP 1. In all cases where the staining in the pelvic connective tissues was positive it was localised to the sub peritoneal collagen layers and fascial bands.

Chapter 7- Growth factors

7.1 Transforming growth factor β

TGF β has important roles in the promotion of tissue repair and regeneration. We sought to determine TGF β activity in the pelvic connective tissues of patients with rectal prolapse compared to control. The control tissue displayed very little TGF β. Amongst the patient groups

the distribution was highly variable. In most cases it was localised to fat cells and the fascial band tissues and those cellular elements that were in immediate proximity to them. Although positivity was seen in the sub peritoneal collagen layer it was not nearly as marked as in the fat layers of some of the patients. Despite this the initial spread of the data was heterogenous (graph 8)



Graph 8 TGF β in fat cells surrounding the rectum

Boxplot showing the spread of data for TGF β in tissues between the patient groups. Statistical analysis using ANOVA and LSD post hoc tests did not show any significant differences between any of the groups.

Although no initial significant results were obtained comparing TGF β between the groups it was noted that there was a wide spread of data points within each group. A sub group analysis comparing disease extent was therefore performed. In this analysis those individuals with external prolapse of the rectum were compared to those who had internal prolapse. It was found that those patients with internal prolapse had significantly more TGF β in their tissues than those who had external prolapse (LSD post hoc test p=0.03) (graph 9)

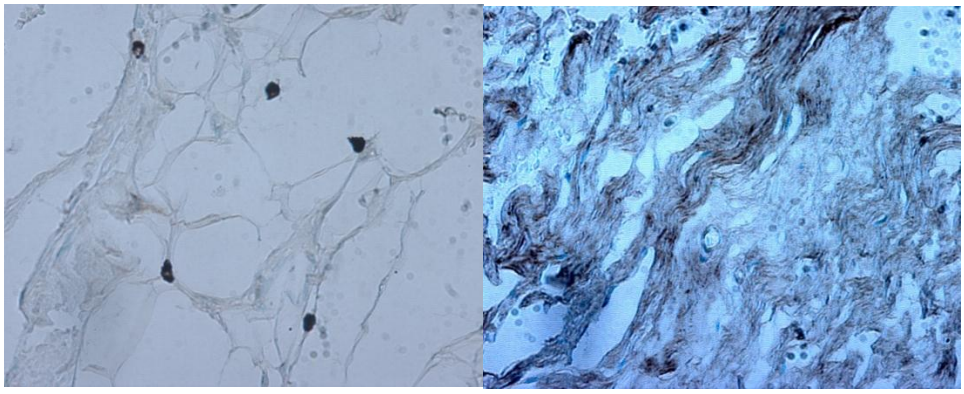
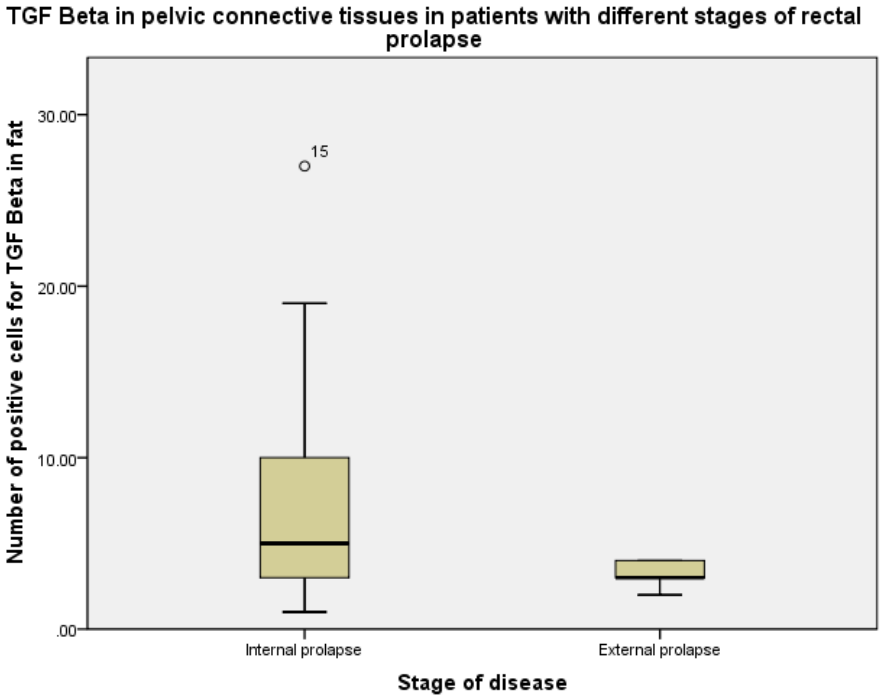


Figure 39 TGF β in tissues and cells surrounding the rectum

Immunohistochemistry for TGF β. Positive cells (stained brown) were typically located in the mesorectal fat (as shown on the left). In others it was located in tissues (right image).



Graph 9 TGF β in fat cells and tissues surrounding the rectum in relation to disease severity.

Individuals with internal prolapse have significantly more TGF β in their pelvic connective tissues than those with the more advanced stage of the disease (LSD post hoc test p=0.03).

7.2 Basic fibroblast growth factor

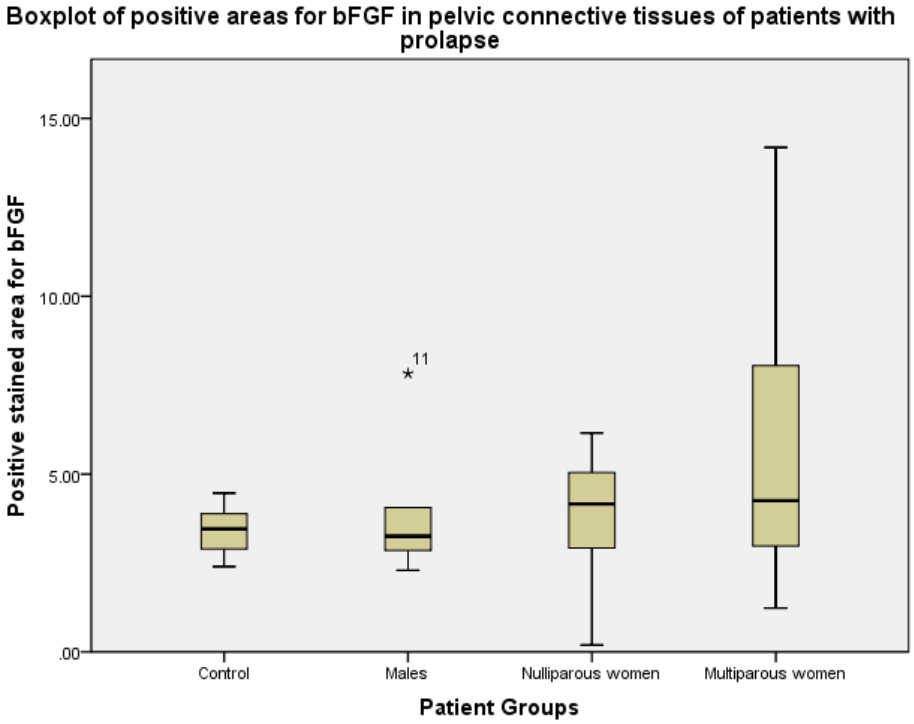
Basic fibroblast growth factor is an important promoter of tissue repair and angiogenesis. We therefore aimed to determine whether bFGF was present in the pelvic connective tissues of patients with prolapse compared with control. Tissue samples were taken from patients with

prolapse and from controls and immunohistochemistry using antibodies targeted to bFGF was undertaken (section 2.12). Results are presented as total area of positive staining for bFGF. There were no significant differences identified between the patient groups with respect to the presence of basic fibroblast growth factor. The results are summarised in table 28 and graph 10.

Table 28 bFGF in tissues surrounding the rectum in patients with prolapse.

Expression of bFGF was broadly similar across the patient groups. For statistical analysis see graph 10.

Group	N	Mean	Median	Range	Standard deviation
Control	5	3.4	3.4	2.4-4.5	0.81
Males	6	3.92	3.25	2.3-7.8	2
Nulliparous women	8	3.82	4.2	0.2-6.2	1.8
Multiparous women	30	4.9	4.1	0.2-14.2	3.05



Graph 10 bFGF in tissues surrounding the rectum

Boxplot illustrating bFGF expression in the tissues of patients with prolapse. Few differences were observed within the groups and no significant difference was noted using LSD post hoc tests and one way ANOVA.

The results were analysed by one way ANOVA and LSD post hoc tests and failed to reach or approach a level of statistical significance, suggesting that basic fibroblast growth factor is not altered in response to the disease process. There were some individuals that appeared to have increased levels of basic fibroblast growth factor. However, no identifiable predisposing feature could be identified to explain these phenomena. Extent of disease was not related and splitting the patients into those groups were both internal and external prolapse was present did not show any overt differences. We have noted that angiogenesis does occur in the pelvic connective tissues of patients with prolapse. It was not possible to determine with certainty whether the microvessel density correlated with bFGF levels. Nor was it intended to examine in detail such a correlation.

Chapter 8- Collagen

8.0 Collagen 1 and 3 in pelvic connective tissues

Collagen is a major component of the extracellular matrix. In order to determine whether there are changes in collagen types 1 and 3 of the pelvic connective tissues with rectal prolapse, samples were taken from the pelvic connective tissues from both patients and controls. Samples were sub grouped according to sex and parity (described in section 2.15).

8.1 Control tissue collagen 1 and 3 types

The pelvic connective tissues in the control groups show grade 3 collagen 1 staining in 67% cases, with grade 2 collagen 1 staining in the remainder. The situation is reversed with collagen 3 expression, which showed grade 3 expression in 33% cases and grade 2 expression in the remainder. The intensity of staining positivity approximates to increased concentration indicating that most of the collagen is of the type 1 variety with type 3 present in reduced proportions.

8.2 Patient collagen 1 and 3 types in pelvic connective tissues

In multiparous women with prolapse the pelvic connective tissues have grade 3 collagen 1 staining in 5% cases, it is grade 2 in 45% with remainder staining grade 1. On studying collagen 3 it was found that grade 3 staining was also seen in 5% cases and grade 1 in 55%, only 45% had grade 2 staining and the remaining 5% none. This suggests that there is less collagen present overall, and that which is present has a ratio of collagen 1 to collagen 3 that is decreased. In nulliparous women with prolapse half the patients had grade 2 collagen 1 staining with a similar proportion having collagen 3 staining of the same intensity. The remainder showed the same pattern with respect to grade 1 staining. Again, this implies a reduced proportion of collagen present overall and an alteration in the ratio of collagen 1 : 3. In male pelvic connective tissues with prolapse 50% had grade 3 collagen 1 staining and the remainder had grade 2 collagen 1 staining. Collagen 3 staining showed that 25% had grade 3 staining with 50% showing grade 2 and the remainder showing grade 1. This suggests that there is more collagen present in the males compared to the multiparous and nulliparous women. It also illustrates a slight deviation away from the control situation with respect to the fact that collagen 3 is present in increased quantities in some patients, whilst at the same time showing a slight reduction in the amount of collagen 1.

Comparison of groups with respect to disease extent, connective tissue status and age failed to demonstrate any meaningful trends or differences.

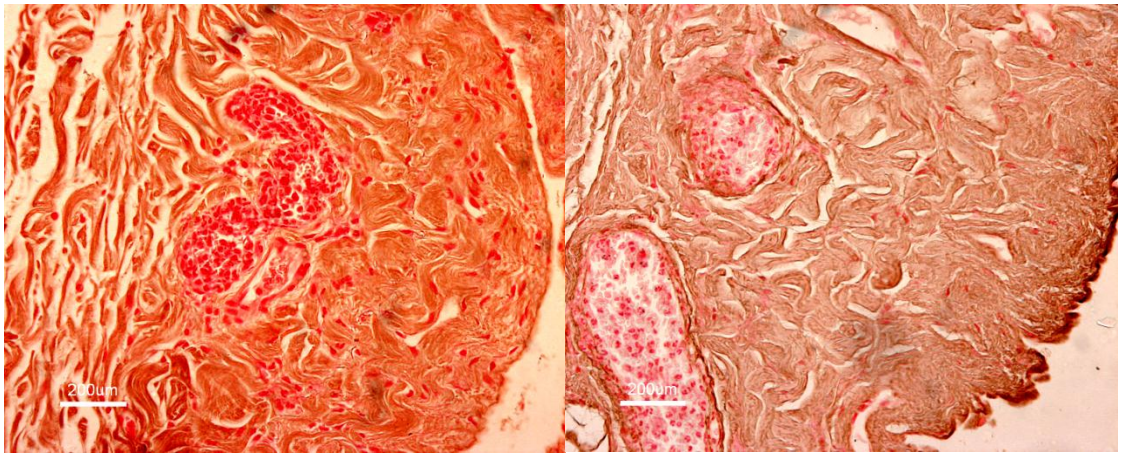


Figure 40 Collagen 1 and 3 in pelvic connective tissues of rectal prolapse patients

Images of immunohistochemistry for collagen 1 and 3 (from same patient). On the left is grade 3 collagen 1 staining and on the right grade 2 staining for collagen 3.

Group	N	Age (Median)	Collagen 1 Grade 1 and 2++	Collagen 1 Grade 3I +++	Collagen 3 Grade 1 and 2 +/++	Collagen 3 Grade 3
Control	5	68	33%	67%	70%	30%
Nulliparous women	6	32	100%	0%	50%	50%
Multiparous women	20	58	95%	5%	95%	5%
Men	6	53	50%	50%	75%	25%

Table 29 Collagen types present in pelvic connective tissues of prolapse patients

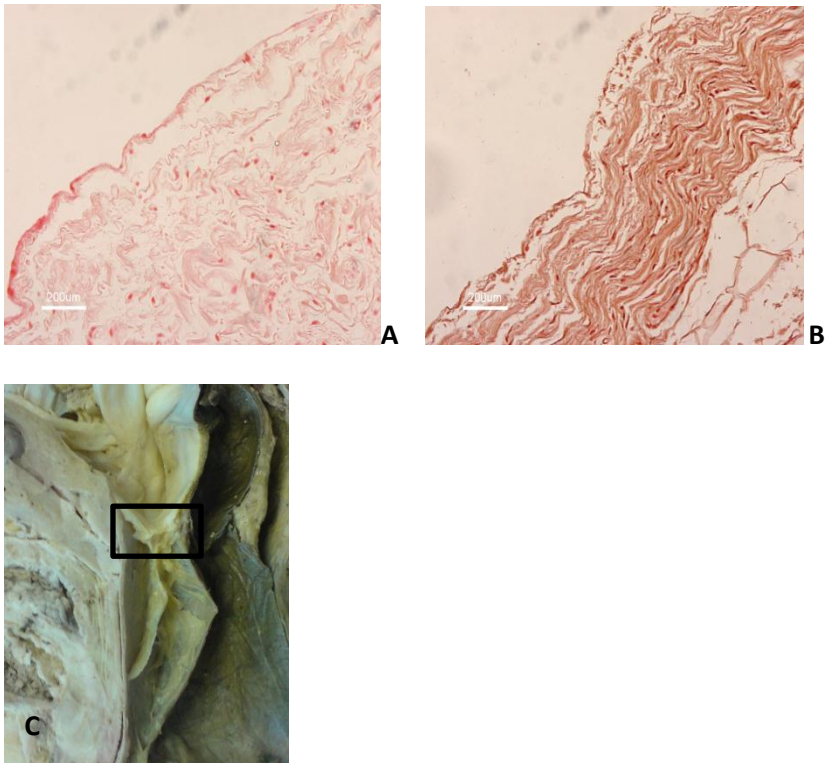


Figure 41 Collagen 1 distribution in patient Vs Control

Sections from multiparous female with prolapse (A) and control tissue (B). The pelvic connective tissues were taken from the region indicated in image C and histologically processed. Sections were stained immunohistochemically for type 1 collagen. In the multiparous female the collagen is structurally disrupted and displays weaker staining for type 1 collagen than the control.

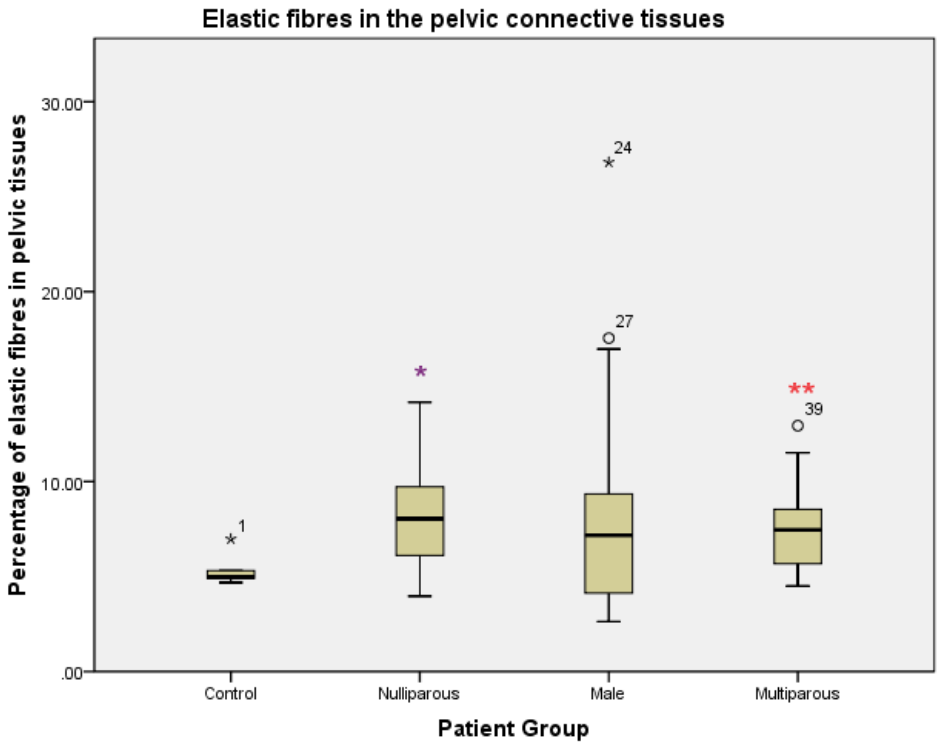
Taken overall, the data does suggest that changes do occur with respect to collagen types in patients with rectal prolapse in terms of overall quantity and also in relation to the proportions of collagen types 1 and 3 that are present.

Chapter 9

9.1 Elastic fibres in pelvic connective tissues

The morphological changes that occur with respect to elastic fibres in the pelvic connective tissues are described in section 3.2. We measured the density of elastic fibres in the pelvic connective tissues of patients and controls with rectal prolapse (described in section 2.17).

As seen in graph 11 there were significantly more elastic fibres in the pelvic connective tissues of the females groups compared to the controls. This was not the case in men; although increased elastic fibres were present in their pelvic connective tissues relative to controls; the differences were not significant (graph 11).



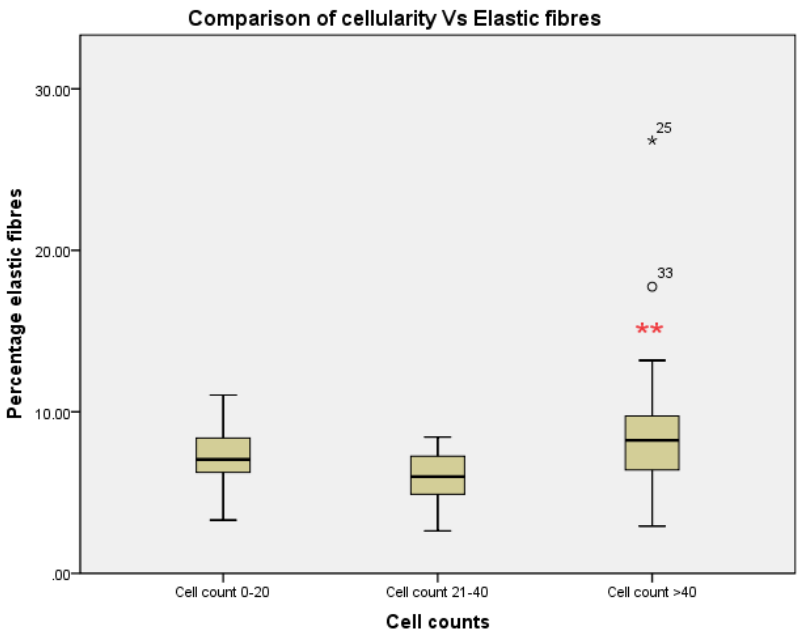
Graph 11- Density of elastic fibres in pelvic connective tissues of patients with prolapse

Elastic fibre density was compared in the pelvic connective tissues of patients Vs controls. The patients were sub divided using parity and sex as differentiators. They tended to have increased elastic fibres. This trend is shown in the boxplot above. ANOVA and Tamhanes post hoc tests were performed. *p=0.02, **p=0.05. The results for the males were not statistically significant p=0.35.

Figure 42 Thresholded image of elastic fibres in pelvic connective tissues.

This is a typical section showing elastic fibre distribution in the pelvic connective tissues. On the left is tissue control and on the right; an image from a multiparous female with rectal prolapse. The sections were stained using an elastin Van Gieson stain (section 2.7). They were digitally photographed and thresholded using Image J (section 2.17) , displaying morphology of elastic fibres (displayed in white) within histological sections. On the left is the relatively normal morphology elastic fibres. On the right increased density elastic fibres displaying evidence of architectural disruption.

To determine whether there was any relationship between cellularity and elastic fibre density samples were taken from several different patients and grouped according to cell counts per high power field using H&E staining. It was found that the patients who had the highest fibroblast counts had significantly more elastic fibres than those with lower cellularity. This finding is in agreement with the observation that patient groups, such as the males which have lower levels of cellularity have fewer elastic fibres.



Graph 12- Relationship between cellularity and elastic fibre density.

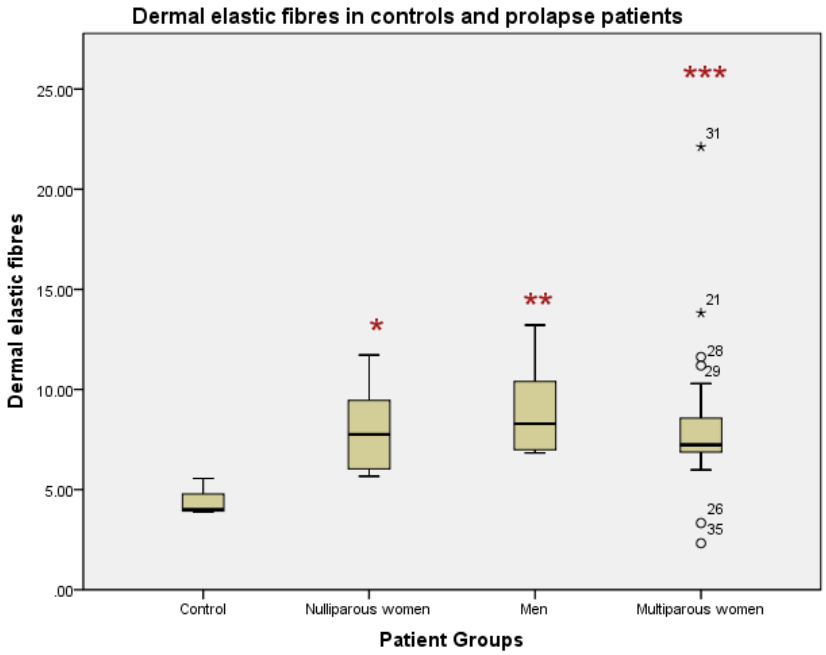
This graph demonstrates the relationship between cellularity of the pelvic connective tissues of patient and elastic fibre density. Samples were taken from the pelvic connective tissues of individuals suffering from rectal prolapse. Samples were grouped according to the number of cells per high power field. Patients whose tissues had the highest levels of cellularity had significantly more elastic fibres than the other groups (p=0.043 LSD post hoc test).**

9.2 Dermal elastic fibres

Although we saw significant differences in elastic fibre density between patients and controls (graph 11) the difference could arise as a consequence of the disease process rather than because of inherent differences in elastic fibre density between patients and controls. We thus evaluated the elastic fibres present in the dermal tissues of patients and of controls (section 2.17). Any inherent disorder of the elastic fibres could also be present in skin (136)

Results showed that dermal elastic fibres were increased in all patients suffering from rectal prolapse (graph 13). The increases were most marked in nulliparous women and males. In the control group the elastic fibres were found to occupy approximately 4% of the dermal tissues, a figure which is similar to that which has been published by others (134). This increased to 7.8% in

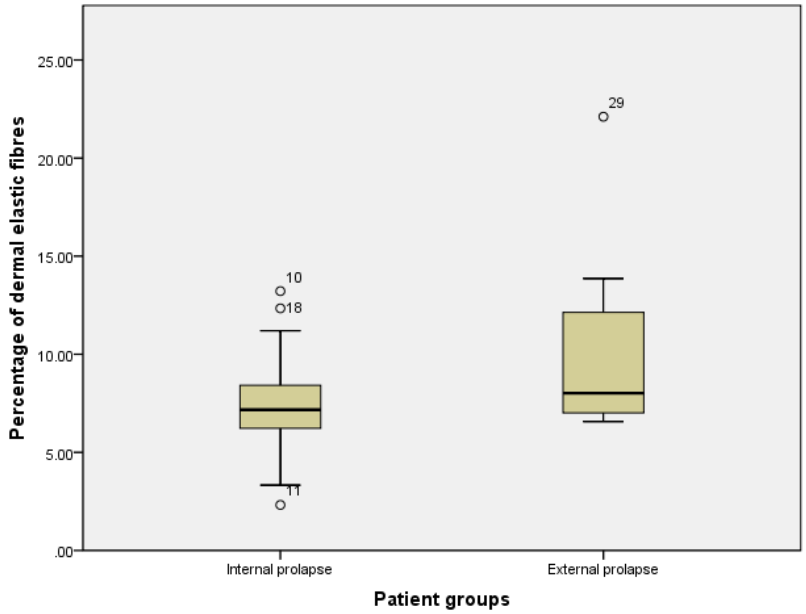
nulliparous women; a similar increase was identified in multiparous women. However, in this latter group the data was more widely spread.



Graph 13- Elastic fibre density in the dermis of patients with rectal prolapse Vs. Control.

In this graph the relationship between dermal elastic fibre density of controls vs patients with rectal prolapse is demonstrated. All groups demonstrated a significant increase in the elastic fibres of the dermis compared with control. T-Tests *p=0.02, **p=0.01, ***p=0.001.

The spread of data in the multiparous group was investigated by sub group analysis. The patients were analysed according to stage of disease (internal vs. external prolapse). It was found that those with external prolapse had significantly more elastic fibres in their dermal tissues than those with internal prolapse (T test p=0.04).

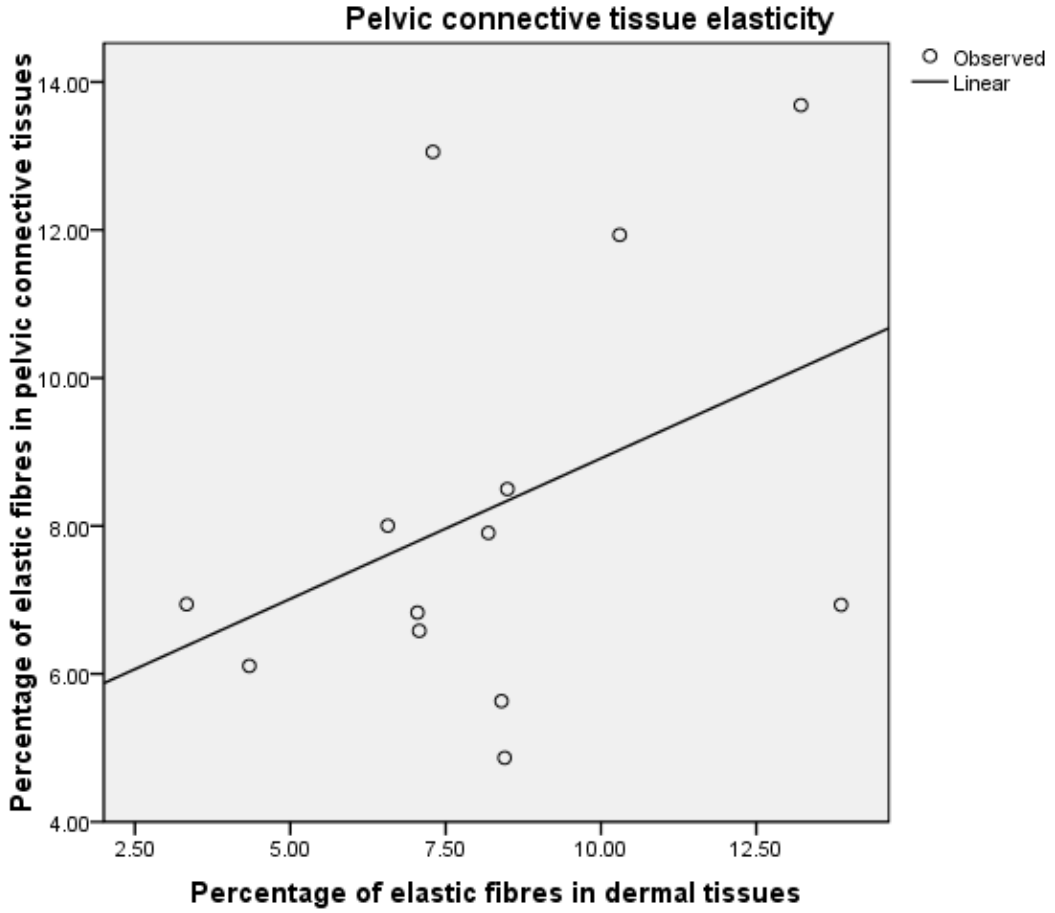


Graph 14 – Elastic fibres in the dermal tissues of patient with internal Vs. external rectal prolapse.

Skin samples were taken from individuals suffering from either internal or external rectal prolapse. The density of dermal elastic fibres was calculated and comparisons made between the two groups. Patients with external prolapse have significantly more elastic fibres in their dermal tissues than those with internal prolapse (T Test p=0.04)

9.3 Relationship between dermal and pelvic connective tissue elastic fibre density

We aimed to determine whether changes in the dermal elastic fibres correlated to changes on the pelvic elastic fibres. The percentage surface area occupied by elastic fibres in samples from pelvic connective tissues and from skin of the same patients was calculated using image J and estimated by thresholding the image as described in section 2.17.



Graph 15 Correlation between pelvic and dermal tissue elastic fibre density.

In order to determine whether dermal elastic fibre density correlated with the density of elastic fibres in the pelvic connective tissues the samples from each site were compared for each patient. Linear regression analysis gave a value of R^2= 0.16. This indicates that there is no significant correlation between the two groups.

When the elastic fibres fibre density of the skin and of the pelvic connective tissues was compared, there was no significant correlation between the two tissue regions. Even after removal of the outliers the R^2 rose only to 0.489.

9.4 Relationship between pelvic elastic fibres and systemic connective tissue status

The percentage elastic fibres in the pelvic connective tissues and the clinical assessment of connective tissue status by Beighton score were compared (section 2.17.3). Patients were grouped into those who fulfilled the diagnostic criteria for benign joint hypermobility syndrome

(n=7) and those who did not (n=8) There was no significant difference between those who had normal connective tissue status (Beighton score 0) and those with clinical evidence of joint hypermobility syndrome (Beighton score >3). In the former group, median percentage of elastic fibres in pelvic connective tissues was 8.5% (5.2-12.9 +/-2.5) in those with hypermobility there was a median of 8.2% elastic fibres (2.6-20 +/-7.5) with no significant differences observed (LSD post hoc test p=0.669).

Chapter 10- Oestrogen

10.1 Oestrogen receptors

The differences seen between men and women patients in distribution of cells and connective tissue components are marked(137). These may have arisen as a result of the effect of oestrogen on the cells in the pelvic connective tissues. It has been previously noted that alterations in oestrogen levels are implicated in the turnover of pelvic connective tissues (138). Oestrogen receptors have been identified in the pelvic connective tissues of those with prolapse and those who do not have the condition, with no overt differences between the two groups (93). We sought to determine whether the density of oestrogen receptors was affected by the prolapse process and also whether males demonstrated this receptor type in their pelvic connective tissues. Samples were taken from the pelvic connective tissues of controls (n=4), males (n=4) and both multiparous (n=12) and nulliparous females (n=5) (methods section 2.14). Oestrogen receptors were greatest in the multiparous women (40 +/-34) and also in nulliparous women (30 +/- 8.8), the males were oestrogen receptor negative (5+/-10) as were the controls (7.5+/-16). The controls were males which may account for the reasons that they were receptor negative. When women were analysed separately, according to age there was no significant difference between those who were older or younger than 45 years (T-Test p=0.71). This data suggests that the female pelvic connective tissues have a basal requirement for oestrogen but in these tissues there is no significant evidence to show that an increase in receptors is present. There were some isolated cases of increased oestrogen receptor status, but it was not possible to determine with any degree of certainty as to why this may occur.

Chapter 11- Discussion

11.1 Clinical features of rectal prolapse

The normal human rectum is a capacitance organ designed for storage. It is supported in the pelvis by a number of connective tissue structures, the most robust of which are the fascial condensations of Waldayer posteriorly (in both sexes) and the Denonvilliers fascia (in men) anteriorly or rectovaginal septum (in women- section 1.2.1)(139). During the course of this research the relationship of the rectum to these supporting structures was delineated. These structures were found to consist of a number of fascial bands which run through the mesorectal fat and provide points of anchorage between the rectum and endopelvic fascia (section 3.1). At the peritoneal reflection a sub-peritoneal structural bridge provides a further link between these structures, the rectum and the endopelvic fascia as a whole (figure 21). This sub peritoneal layer is usually overlooked in surgical circles as it does not lie within the standard planes of surgical approach to the rectum for cancer surgery. As in other human anatomical studies, unravelling the anatomy has been hampered by access to insufficient numbers of patients and variability in dissection methods. Furthermore, the tendency to focus on the development of gynaecological organ prolapse has overlooked the potential importance played by this structure in supporting the rectum. As a result of this research the existence of this structural network is evident and clearly apparent in all cases studied, be they cadaveric, control or patient (section 3.1). Confluent with this structural network superiorly is the sigmoid mesocolon which serves to support the distal sigmoid colon (figure 8).

During the development of rectal prolapse the structures which support the rectum fail. As a result a number of different clinical sequelae may ensue. Proximal failure may manifest itself as recto-rectal intussusception (internal rectal prolapse) in which the proximal rectum migrates distally (figure 12). An alternative event is that local failure of structural support at other sites, most commonly at the rectovaginal septum, can result in anterior protrusion of the rectum into

the vagina, a rectocele. When this occurs in the subperitoneal collagen layer the small bowel, which often resides in the pelvis, may protrude inferiorly to compress the rectum, an enterocele (figure 29). These site specific failures may occur in isolation or in more advanced cases as part of a global failure of pelvic floor support in which case all of the above may be present. As the condition progresses, the rectum may protrude externally manifesting as a full thickness external prolapse. Historically, it is this advanced form of the disease that is best described(4), not least because it poses an immediate clinical problem. The internal phenomena have been relatively poorly described until recently, because of a combination of poor understanding of the condition and lack of comprehensive patient diagnostic investigations (table 7).

Patients with the internal disease variants may present with a bewildering array of non-specific symptoms and physical signs. These range from obstructed defecation through to chronic pelvic pain. A striking feature in this patient group is the clinical improvement in symptoms which occurs if these conditions are accurately diagnosed and appropriately treated(140).

11.2 Macroscopic features of disease

Patients that come to surgery for rectal prolapse have characteristic findings associated with the condition. These include a tendency to have a hypermobile colon, peritoneal defects, hypermobile pelvic connective tissues, occasional pelvic free fluid and peritoneal defects (section 3.2). One of the main difficulties lies in defining these features objectively. However, in the small series that were prospectively evaluated a pelvic caecum could be clearly identified in over 60% of cases and the true figure may be even higher(section 3.2). Causal observation indicated that an excessively redundant sigmoid colon was also present in the majority of cases. However, it was difficult to define this objectively and therefore this was not pursued. The excessively mobile colon may be indicative of a generalised connective tissue defect that will be considered elsewhere. Peritoneal defects, including evidence of enterocele could be identified in over 40% of cases (section 3.2). Whether these are the cause of the condition or occur as a result of it are

unclear at the present time. The peritoneal defects, particularly enterocele, are of considerable clinical importance since they restrict the surgical options available to patients(141). Lack of awareness of enterocele may have catastrophic consequences should surgeons attempt to perform a stapled transanal resectional rectopexy(141). These macroscopic internal features correlate with disease manifestations that have been described by others, over 40 years ago Parks commented on patients with a disease referred to as “descending perineal disorder”(4), which is most likely to represent internal rectal prolapse. In view of the internal macroscopic features of these disorders accompanying pelvic floor failure, the development of a patulous pelvic floor is likely to represent the external manifestation of this process.

11.3 Microscopic changes in pelvic connective tissues of patients with prolapse

The finding of overt macroscopic evidence of a disease process led to the detailed study of the tissue changes occurring in response to the process of rectal prolapse, which constitutes the bulk of this thesis. One of the disadvantages of studying these changes histologically is that the tissue changes for a single patient are captured only at a single time point in the sequence of disease progression. It is necessary to make some inferences of the changes that are seen to enable conclusions to be drawn about potential processes involved in the progression of the condition.

Detailed study of cadaveric material and microscopy of control samples confirms the existence of the connective tissue supports that are visible macroscopically (section 3.1). In the normal situation the topography of these tissues is quite characteristic, these supports consist of collagen fibres, elastic fibres and fat (figure 21). The arrangement of the sub peritoneal collagen layer, which in this study was deemed to be representative of the pelvic connective tissues as a whole, demonstrated that the collagen fibres were arranged in a criss- cross pattern . The elastic fibres of this layer appeared to form part of this mesh with projections superiorly to the collagenous layer and inferiorly into the fascial bands (figure 27) that traverse the mesorectal fat and ultimately attach to the rectum and pelvic side walls (figure 21).

In patients with rectal prolapse there is considerable disruption to the sub peritoneal collagen layer (figure 33). Less than 50% of patients in the sub groups studied had any evidence of architectural normality to this layer (table 20). Damage was most marked in multiparous women, perhaps as a result of the shearing forces in the pelvic connective tissues that occur during parturition. Similar changes were also noted in men and nulliparous women, indicating for the first time that perhaps obstetric trauma is not the sole event in the development of rectal prolapse. The method of tissue collection may be argued to be a cause of the disruption to the collagen layer that was seen. However, in most cases the morphology of the collagen fibres was altered and where fragmentation of mature fibres had occurred there was usually evidence of collagen with a different morphology present in the tissues, such changes would go against simple mechanical disruption incurred during the process of tissue retrieval. The changes in the collagen layer were variable, in multiparous women it was usually thickened, although the collagenous structures were disorganised. Microscopically this was most analogous to scar tissue that is seen at other sites and may therefore represent attempted repair

The finding of elastic fibres in the disordered collagen layers suggests that any reparative process would have occurred many months or years previously because elastic fibres are only laid down during the latter stages of the healing process(102). The cells involved are usually myofibroblasts; these were found in these tissues (section 5.3) and will be discussed further later. The absence of immature collagen fibres (figure 33) suggests that these are either not being formed, or being degraded once they have been formed.

In a small proportion of cases the phenomena of fat herniation was identified (table 19), here fat cells were able to penetrate a disrupted collagenous supporting layer. These changes were always associated (table 19) with changes in the elastic fibre layer which, too, would show evidence of structural disruption (figure 33).

In histological sections the proximal migration of fat cells is most likely to result in the development of an enterocele, this is because the normal structural components separating the bowel from the mesorectal fat are missing. The problem may be propagated, in part, because the enterocele may compress the rectum resulting in obstructed defecation, patients then strain to evacuate their rectum, often for prolonged periods, in women with uterine prolapse this may cause the prolapse to extend(142). The anatomical defects associated with rectocele may have other effects because they are part of the tissues that support the anterior rectal wall. Where these supports are damaged, the anterior rectal wall may then tend to fall posteriorly into the rectal lumen (figure 44). A combination of repeated straining and gravity may then serve to propagate the intussceception, this may in turn cause further traction on the pelvic tissues.

Biomechanical studies were not conducted formally as part of this research but observational evidence of hypermobile pelvic connective tissues can be seen in many patients with prolapse (table 19) and therefore suggests that these changes may well have some biomechanical sequelae.

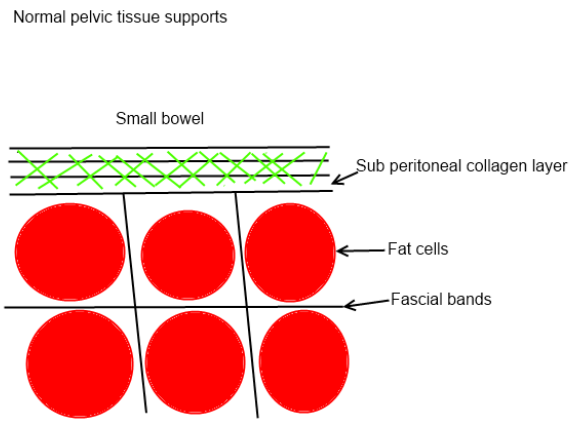


Figure 43 Topography of normal pelvic connective tissues

Schematic showing the topography of the pelvic connective tissues in a normal individuals. Collagen fibres are marked in black, elastic fibres are green and fat is red. Note how there is a clear demarcation between the fat layer and the collagen layer.

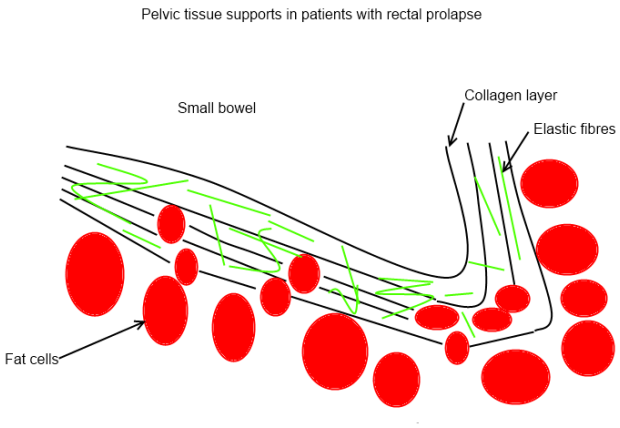


Figure 44 Pelvic floor connective tissues in the disease state

Schematic diagram demonstrating the changes that occur in the pelvic connective tissues in association with the development of rectal prolapse and enteroceles. Note how the collagenous layer (black lines) is disrupted with fat cells contained within it. This leads to morphological changes in the pelvic connective tissues with recession of the Pouch of Douglas, as demonstrated above.

11.4 Collagen in pelvic connective tissues of patients with prolapse

Collagen is an important component of the extracellular matrix. There are over 25 different types, each type being encoded by a different gene product. The collagen types present varies from tissue to tissue according to function. Collagen type 1 is the most ubiquitous, forms fibrils and is the major collagen of bone, tendon and skin. In many tissues there are both 1 and 3 type fibrils present, the combination of collagen types 1 and 3 is said to confer properties of increased flexibility on different tissue types (though this statement is largely based on studies detailing the collagen types that are found in different tissues rather than by direct analysis). In disorders such as stress urinary incontinence(33), haemorroidal disease(40) and uterine prolapse(55) the proportion of collagen fibrils 1 and 3 is altered (table 1) in pathological relative to normal tissues. Changes in collagen types at the gene level have also been undertaken by performing mRNA studies(143).

We hypothesised that in rectal prolapse as seen in other pelvic floor disorders, the distribution of the collagen types would be altered. Results were in agreement with previously published data on collagen distributions in pelvic connective tissues(144). In patients with rectal prolapse there is less collagen in the pelvic connective tissues compared to controls, and the ratio of collagen 1 to 3 is reduced (table 29, section 8.0). This pattern was present in multiparous women and may be the result of increased amount of scar tissue in their pelvic connective tissues. The nulliparous women show further decreases in the amount of type 1 to 3 collagen relative to controls, this group also show evidence of structural injury. It is difficult to make a link between collagen sub type and function, it is not possible to state with certainty that these changes explain the physical changes in increased mobility of tissues at the site of disease, but they are suggestive of it. .Although the changes in rectal prolapse tissues is similar to that seen in other analogous processes, there are problems in extrapolating and comparing other results to the rectal prolapse setting. Partly because the methodologies have been used in each case setting. Attempts were made in this study to quantify the amount of collagen present using a hydroxyproline assay. However, the presence of fat in the samples coupled with the difficulties of sampling small pieces of tissue led to results that were non reproducible and therefore these are not presented.

11.5 Benign joint hypermobility syndrome and the relationship to prolapse

The alterations in pelvic collagen in patients with rectal prolapse may be indicative of a localised or generalised disease process. Evidence of the latter is provided by those individuals with BJHS (table 21, section 4.0). It is clear that those with BJHS present in a different manner to those patients who do not have the condition (table 21).

The most striking finding was that those with BJHS presented at a significantly younger age (9 years younger) than those without the condition (table 21, section 4.1). It is probable that those with BJHS developed the pelvic floor disorder at an even younger age, since we measured age at presentation for surgery at our unit, rather than age of first developing symptoms or of

undergoing a previous surgical procedure that may be related to prolapse. The reasons for this are due to the fact the surgeon performing the initial operation may not have realised that an underlying rectal prolapse was present. Although this is clearly not the case if the procedure was in itself an anti- prolapse operation. These patients also tended to have more attempts at surgical correction of prolapse (with subsequent failure) and need for revisional surgery (table 22, section 4.2). Patients records show that those with BJHS who had previously undergone treatments at other centres, had had a heterogeneous series of surgical procedures performed. In some cases attempts had been made to correct the prolapse with a STARR procedure or procedures designed to treat haemorroidal disease (when in fact a prolapse was present). The high rate of previous attempted surgical intervention in those who have BJHS may account for the reason why the disease extent in those with BJHS was not significantly different from those without the condition. During the timeframe of the study it was not possible to recruit sufficient numbers of patients with BJHS who had not undergone previous treatments into order to address the issue of disease extent at presentation in a conclusive manner. Indeed, this question is not of much clinical importance and therefore does not justify the time, effort and resources that would be needed to answer it.

Since those individuals with BJHS presented in a different manner (table 19) from those without the condition we then sought to evaluate their surgical outcomes. We have long observed that the outcomes following surgical treatment of rectal prolapse are highly variable and many operator and patient factors have been cited as reasons for treatment failure. As a tertiary referral centre for pelvic floor disorders we have considerable experience in the treatment of rectal prolapse and patients with established rectal prolapse will usually undergo laparoscopic ventral mesh rectopexy. This is a safe and effective treatment for most patients (145). In this latest study we found that those individuals presenting with rectal prolapse and who had normal connective tissue status had a minimal requirement for revisional surgery in the 12 months after their primary procedure. However, in those with BJHS recurrence of the condition resulting in re-

operation was noted in 25% of cases (section 4.2, table 22). Whilst this is not an unacceptable rate of recurrence following rectal prolapse surgery (some procedures have a 50% failure rate at 5 years(21)), it does help to explain why some individuals do less well.

Individuals with BJHS have an increased risk of developing a range of pelvic floor disorders including stress urinary incontinence and disordered colonic transit(116-119). Those developing the former are indicative that there may be a global failure of pelvic floor support. In the latter groups it may be that rectal prolapse was present from the outset, in a published series from Oxford, rectal hyposensitivity was found to be rare and most cases did have clinical evidence of an underlying anatomical explanation for their symptoms(146). However, it is possible that in some patients there may also be neuronal effects resulting or contributing to the condition. There are some well reported neuro-psychiatric phenomena associated with BJHS including abnormally low pain thresholds, resistance to the action of local anaesthetic agents and mood disturbances(147). By virtue of the flexibility these individuals often excel in the performing arts and sports and in one published series up to 50% of patients attending pelvic floor clinics in London were found to have BJHS(115). Further indirect evidence towards a neuronal contribution is provided by the response of some of these patients to sacral neuromodulation. The pattern of disease recurrence in those with BJHS was consistent; symptoms generally occurred approximately eight to twelve weeks following the initial procedure. On investigation the site of recurrence was usually the posterior rectum. This is interesting since the posterior rectum is supported by Waldeyers fascia, a strong fascial condensation linking the rectum to the sacrum(11). This region is rarely damaged during processes such as parturition and is not a common site of recurrence in those who do not have BJHS. It is continuous with the connective tissues sampled in the study so there may be similar biological factors at play. However, it is equally possible that these tissues are intrinsically abnormal in those with BJHS. Traditional operations for rectal prolapse formally dissected these tissues in the posterior plane and re-enforced them with prosthetic meshes. However, these procedures were marred by a range of

poor long term outcomes, including erosion of the meshes into the rectum with resultant chronic sepsis(148). The posterior rectal mobilisation required to allow the placement of a mesh in this plane was associated with a high risk of autonomic nerve injury with associated effects on rectal physiology. A newer technique of modified Orr-Logue rectopexy has been devised to specifically address this issue. It will be interesting to determine whether this improves the outcomes in those with BJHS. One subgroup of participants whose data was not presented was those who had some clinical evidence of increased tissue flexibility (resulting in Beighton score of 1 or 2). Individuals in this category could only receive a diagnosis of BJHS if they fulfilled a number of symptomatic criteria (mainly musculoskeletal), these individuals were not encountered in the study population. However, analysis of outcomes in participants with evidence of increased flexibility falling short of BJHS did not show any significant differences compared to those with normal tissue status. In this particular group it is possible that these individuals were slightly more hypermobile because they were younger and it is well recognised that younger individuals typically display greater tissue mobility than that seen in older individuals. Overall, the data relating to BJHS demonstrates a cautionary note for surgeons in the speciality, for they undoubtedly do less well with interventions, and where surgical treatment is needed a prosthetic mesh may be the safest method of surgical reconstruction.

11.6 Elastic fibres in pelvic connective tissues and skin of patients with prolapse

Elastic fibres form an important component of the extracellular matrix(45). They confer upon tissues the properties of recoil and resilience(149). They are ubiquitous and their function is best exemplified by considering blood vessels where they form a discrete connective tissue layer and where their involvement with diseases such as Marfans syndrome (a genetic condition affecting the fibrillin 1, the major microfibrillilar protein of the elastic fibre network(150)) has significant long term complications such as the development of aortic aneurysms(151). Pelvic organ prolapse is well recognised in those who suffer from conditions such as Marfan syndrome(152), in animal

models designed to study prolapse, deletion or alteration of microfibrillar proteins can lead to rectal (and uterine) prolapse either early post-partum or with the passage time in nulliparous mice(49).

In humans, elastic fibres are laid down late in foetal development and are not usually subjected to the same tissue turnover processes as other components of the ECM(153). It has been recognised for some time that the rectal mucosa in patients with rectal prolapse is characterised by the deposition of increased quantities of elastic fibres and the morphology of these elastic fibres is quite different to those which are seen in non-diseased setting(53). Because elastic fibres are typically laid down in foetal life, but are also laid down in wound healing(47), they presented a potentially useful method for assessing changes in the pelvic connective tissues during the development of the pathology and also in the periphery. This is because in the non diseased setting the morphology of these fibres should be generally conserved.

In section 3.2.2 the morphological changes affecting the elastic tissues were described. In general terms conditions such as enterocele were associated with disruption to the elastic fibre layer. In many cases there was evidence of increased deposition of immature appearing elastic fibres in those areas where defects in the pararectal connective tissues were identified (figure 33). The changes in the elastic fibres were most marked in multiparous women who showed the greatest increase in elastic fibres of their pelvic connective tissues. Whilst it may be tempting to attribute this exclusively to the effects of childbirth it should be noted that increases in the elastic fibres of pelvic connective tissues in nulliparous women were also identified (graph 11, section 9.1). In this group obstetric trauma has not occurred and therefore these tissues are either disrupted during the process of prolapse or contribute to its development. There was a relationship between elastic fibre density and cellularity (considered in section 9.1). This suggests increased synthesis of elastic fibres in the tissues of some patients with rectal prolapse compared to others. Males with rectal prolapse do not display this increase in elastic fibres, although evidence of structural

disruption was certainly present (graph 11). The changes in elastic fibres in male pelvic floor disorders have not been previously studied. Studies conducted in animals and females with pelvic organ prolapse have yielded conflicting results. With regards to elastic fibres, decreases have been identified in association with prolapse of the uterus (as seen in the male patients – graph 11, section 9.1)(41). Increases in elastic fibre density are less widely reported, this may reflect differing techniques, since many of these studies use desmosine assays to determine concentration of elastic fibres and hence are unable to localise the changes. When fibulins, which are co-localised to elastic fibres(45), are considered, decreases are seen(49, 154, 155). These fibulin studies tended to use mRNA expression to characterise changes; results may not directly relate to protein expression and hence elastic fibre density.

Elastic fibres in the pelvic connective tissues may be subjected to a number of processes intrinsic to the development and progression of the disease. To determine whether these increases that were noted were part of a localised or intrinsic difference relative to control tissue, it was decided to study the elastic fibres in skin, as this tissue is readily accessible, not involved in the disease process and regarded as “a window into our heritable diseases” (156). The technique for quantifying elastic fibres worked well (134) and gave clear reproducible results, the normal form and distribution of elastic fibres in the dermal structures of the skin was in agreement with findings described in the literature(134). In individuals with prolapse it was found that there were increases in the percentage of dermal elastic fibres in all groups (graph 13, section 9.2). In the multiparous group there were wide spread data points, the elastic fibre density in these patients was then evaluated with respect to disease extent. When the multiparous group was split in this way it was found that those with external prolapse had a slightly higher percentage of dermal elastic fibres than those with the less advanced stage of the disease (graph 14, section 9.2). The differences were not large (1%) but did achieve statistical significance (and perhaps clinical significance as well). The findings of increased dermal elastic fibres are difficult to explain. None of the patients had overt evidence of an elastic fibre disorder, such as Marfans syndrome. The

morphology of the dermal elastic fibres, unlike those of the pelvic connective tissues was relatively normal. What is unclear is whether the alterations are indicative of an underlying elastic fibre disorder or the consequences of a genetic disorder of another component of the extra cellular matrix, tenascin X deficiencies, for example, can result in abnormal elastic fibre formation(157).

It is highly likely that rectal prolapse is a multifactorial disorder and it may be that this is little more than a single component of the process. We sought to determine whether there was any direct correlation between dermal elastic fibre density and that of the pelvic connective tissues, even when major numerical outliers were excluded a linear relationship could not be established (graph 15, section 9,3) and an R^2 of 0.48 suggests that it is not possible, at this stage, to use dermal elasticity as a proxy marker for the situation in the pelvic connective tissues. In view of the dynamic changes in the pelvic connective tissues themselves it is perhaps not unsurprising that they do not correlate directly.

11.7 Cellular changes in pelvic connective tissues in patients with prolapse

The structural changes observed in the pelvic connective tissues are analogous to those which are seen during wound healing. There is evidence of tissue disruption, attempts at repair and tissue breakdown (section 3.2). In a human wound there is a carefully orchestrated sequence of cellular proliferation, matrix synthesis and remodelling of the extracellular matrix during which time the cellularity of the tissues declines(102). This sequence of experiments had a number of aims, to characterise the basal cellular population in the pelvic connective tissues and to assess how this changed in response to rectal prolapse.

In the controls, the pelvic connective tissues contain a resident population of fibroblasts (figure 35, section 5.1). The overall cellularity of the control tissues is low (graph 1, section 5.1), as would be expected from the wound healing model. This resident population of fibroblasts virtually all show markers distinguishing them as myofibroblasts. These cells have actin fibres distributed

within them which confer upon the tissues a contractile phenotype. During the process of rectal prolapse a number of changes occur. Most immediately apparent, particularly in multiparous women, is the increase in cellularity (table 23, section 5.2). This process is similar in nulliparous women, and to a much lesser extent in men with prolapse. The experimental data supports the hypothesis that this increase in cellularity is brought about by proliferation of this resident cellular population, rather than migration from surrounding tissues. There was a trend to increased cellularity in those with external prolapse relative to internal prolapse (table 24, section 5.2). However, this was not statistically significant. Clinical observations suggest that internal and external rectal prolapse are part of the same disease process and therefore the lack of difference in cellularity between these two stages is not unsurprising. When elastic fibres were studied those patients with the greatest increase in cellularity showed an increased elastic fibre density of the pelvic connective tissues (graph 12, section 9.1). In view of the immature morphology of these fibres it would seem plausible that the fibroblasts are synthesising elastic fibres. Another structural feature of the process of rectal prolapse is the increase in the number and thickness of fascial bands between the rectum and pelvic sidewall (section 3.2). Cellularity was not found to relate to this in the same way, although it is likely that these bands are synthesised by fibroblasts in response to the insult of prolapse. When the phenotype of these cells were evaluated it was found that in nulliparous women the cells differentiated to become myofibroblasts, this was not the case in multiparous women, where the fibroblasts did not display the same marker of differentiation. In men, far less proliferation was seen and the proportion of myofibroblasts remained high, probably because the cells were not proliferating (table 26, section 5.3). These intersex differences are of potential clinical importance, because they indicate that once the process of rectal prolapse is initiated in males, very little cellular response occurs. This may translate into a limited capacity for improvement in tissues with non operative management because the resident cellular population does little to attempt to effect a repair or regenerative process.

Since the myofibroblasts have a contractile phenotype the lack of differentiation into this cellular type in some patients may be significant because it may help to contribute to increased flexibility of the pelvic connective tissues at points of mechanical weakness that result from pelvic floor trauma. These cells are unlikely to be a major contributor to the disease process in their own right, as nulliparous women demonstrate the process of differentiation but still have the disease. The lack of myofibroblasts in the pelvic connective tissues of some patients may be surgically important because they are an important component of the healing process in traumatised tissues. In incised dermal wounds they produce wound contraction during the latter stages of wound healing(102). This process is coupled with collagen remodelling so that collagen fibres in dermal wounds are aligned to support areas of increased tension. If this process does not occur in the pelvic connective tissues then remodelling may not occur in the same organised manner. When rectopexy (without placement of mesh) is performed, up to 30% of patients may suffer from recurrence of the disease(21). This could be reflective of inadequate fibrosis surrounding the rectum in these patients, a process that is myofibroblast dependent.

11.8 Matrix metalloproteinases in pelvic connective tissues of prolapse patients

In view of the cellular (section 5.0) and morphological (section 3.2) changes identified in prolapse tissues it was hypothesised that MMP's would be expressed in the tissues as these enzymes are integral to the degradation of components of the extracellular matrix. In total four MMP's were studied. The most marked changes were seen with MMP 1 (section 6.1). Unlike the other MMP's that were studied, the hemopexin domain that is present in MMP 1 renders it capable of cleaving and degrading fibrillar collagens. It was principally located in the collagenous tissues surrounding the rectum and was not identified in the mesorectal fat cells. This may reflect the sample preparation methods since the fat content of adipocytes is removed during paraffin processing. The tendency for MMP1 to be present in the tissues indicates that it is located in a region where

it will exert the greatest biological effect. The concomitant absence of TIMP 1 expression (section 6.6) renders it potentially biologically active. Proof of this latter point is provided by the absence of fascial bands noted in the mesorectal fat of some of the prolapse patients with the most intense staining for MMP1 (section 6.1). It is likely that the increase in MMP 1 may contribute to the progression of the disease by virtue of is degenerative activity in excess of the ability of surrounding tissues to synthesise sufficient components of the ECM to replace that which has been degraded. This process has been associated with the progression of prolapse in patients with uterine prolapse (71-73, 158-160). What is unclear is whether the prolapse occurs as a primary event with MMP's released as a secondary phenomena or whether MMP 1 is released early in the disease process and contributes directly to tissue damage. There is some evidence to suggest that tensile and shearing forces may lead to MMP 1 release(161). One of the difficulties is that these studies have been largely conducted in tendinous tissues so are not completely analogous to this situation.

The MMP's 3, 7 and 9 all differ from MMP 1 in that they cannot degrade fibrillar collagen. They cannot therefore initiate collagen breakdown. Increases in MMP 9 were seen (section 6.5) and it is likely that this works in association with collagen 1 in remodelling the extracellular matrix. It is also inhibited by TIMP 1 and therefore the absence of this inhibitor in many of the tissue samples is likely to render it (MMP 9) biologically active. This is concordant with findings in uterine prolapse (and other similar disorders) in which increases in MMP 9 have been identified (28, 29, 49). The results relating to MMP 3 showed that the greatest increases occurred in nulliparous women. In the other groups less marked expression was seen (section's 6.3 and 6.4). The expression of MMP 3 in relation to other prolapse disorders is less extensively studied. Investigators studying the change in MMP3 mRNA expression in uterine prolapse, saw only a slight trend towards increased expression (143).

Taken overall the combination of increases in MMP and decreases in TIMP would seem to create a potently proteolytic environment in the pelvic connective tissues of some patients. This, in turn, may lead to more rapid disease progression. Where less MMP 1 is identified, the process of matrix synthesis appears to keep pace with degradation, and the more expected appearance of increase ECM components is seen (section 6.1). It should be remembered that alongside this are the on-going tensile forces transmitted through the pelvic floor during repeated valsalva manoeuvres(142).

11.9.0 Growth factors

The processes involved in tissue repair and regeneration are partly controlled by a series of growth factors. Of these, transforming growth factor β and fibroblast growth factor, are probably the best recognised, and, arguably the most important.

11.9.1 Fibroblast growth factor

In wounds basic fibroblast growth factor is released by fibroblasts and is responsible for inducing fibroblast proliferation and local collagenase release (162). It was hypothesised that an analogous situation may occur as a result of the injury to the pelvic floor connective tissues. This view is supported by the increases that were noted in MMP 1 (table 27, section 6.1). However, there was no apparent difference in FGF 1 levels amongst the patient groups (table 28, section 7.2). The multiparous women showed a trend to increased bFGF, however, this was not statistically significant. Although bFGF appears to play an important, but poorly understood role in wounds at other sites; the experimental data obtained here indicated that bFGF is not important in the process of rectal prolapse development. In wounds at other sites, the primary event is one of blood vessel disruption resulting in a fibrin rich clot that must be transformed into a collagen rich matrix in order for wound healing to proceed. If the process of vaginal delivery, or the initiation of prolapse was considered as the primary wounding event then it would be unlikely for it to be sustained and data derived from human wound healing studies suggests that bFGF levels rise and

fall as healing progresses (163). This may be another reason in explaining the observations noted in our samples.

11.9.2 Transforming growth factor β

Transforming growth factor β has a range of biological effects including fibroblast differentiation and matrix turnover. It is well described in human wound healing as inducing connective tissue synthesis(164, 165). We therefore hypothesised that in our patients with prolapse we would expect to see increases in TGF β proteins in the pelvic connective tissues of patients suffering from prolapse. In our patients with prolapse there was a trend towards an increase in TGF β amongst those suffering from prolapse (graph 8, section 7.1), though this did not reach a level of statistical significance. In general, TGF β was identified in the cells of the mesorectal fat and it is possible that it may serve a role in the development of the fascial bands that traverse this layer in response to the prolapse process, particularly since it is suggested that it may decrease MMP activity. The lack of marked increases in all groups as whole was against our anticipated hypothesis and is also against reports from in situ mRNA studies performed in the women with stress urinary incontinence where increases were noted(166). However, in uterine prolapse it has been noted that a decrease in TGF β was identified in individuals with prolapse (88). This is in agreement with our data in relation to the stage of disease in multiparous women, where a decrease in TGF β expression was identified in those with external prolapse (graph 9, section 7.1). To date no studies have evaluated the role of TGF β in rectal prolapse disorders. However, it would seem that in response to rectal prolapse slight increases in TGF β are seen and that this is greatest in those with internal prolapse (where it may serve a protective function), in those with external prolapse it is present in lower amounts and this may contribute to the failure of the pelvic connective tissues in this group by allowing unopposed tissue breakdown.

11.10 Oestrogen receptors

Female pelvic floor disorders, particularly in those women who are perimenopausal have been treated with topical oestrogen preparations for many years(97). Many of these treatments have

been anecdotally reported as being beneficial. We thus sought to determine whether oestrogen receptors were present in the pelvic connective tissues of patients with prolapse and in controls. In addition we also sought to determine whether male pelvic connective tissues displayed any evidence of possessing oestrogen receptors.

Increases in the oestrogen receptors of the multiparous women were greatest, although increases were also noted in nulliparous women (section 10). The lack of an increase in oestrogen receptors in males was not unsurprising. In gynaecological prolapse, an increase in oestrogen receptors of pelvic connective tissues was correlated with an increase in serum oestrogen levels in pre-menopausal women(167), they also noted an increase in oestrogen receptors in post-menopausal women. We saw a trend towards this. However, when age (which was our best marker of menopausal status) was compared across the groups there were no differences noted in relation to oestrogen receptors. This would suggest that in rectal prolapse, in contrast to gynaecological prolapse, the roles played by oestrogen are less significant. Indeed other researchers have also demonstrated that the effects of oestrogen increases in the setting of pelvic floor disorders are heterogeneous and occasionally worsen the disease process(168, 169). From the data that we have obtained, it would seem unlikely that administration of exogenous oestrogens would be helpful in the treatment of rectal prolapse disorders.

Chapter 12- Conclusion

Rectal prolapse is a complex multisystem disorder. At the outset of the study the pathophysiology of the condition was poorly understood. A variety of different patients may present with the disorder and there may be aetiological overlap, accounting for the condition in different individuals. On the basis of the data presented it is evident that a subgroup of patients have clinical evidence of a systemic tissue disorder and that these individuals fare less well with surgical interventions than others, with normal connective tissue status undergoing the same procedure. This is concordant with previous work which has demonstrated highly variable outcomes following surgery for rectal prolapse and may provide some explanation for the results that are detailed in the published literature.

This thesis has also produced experimental data illustrating a difference in dermal elastic fibres present in many of the patients who present with rectal prolapse. Until now individuals presenting with rectal prolapse were widely cited as having a weak pelvic floor, caused by obstetric trauma. By analysing patients in whom this process is absent it is possible to demonstrate a difference in dermal elastic fibres and also to demonstrate that this is also present in a subgroup of those who do have an obstetric history. In this latter group the changes are particularly noteworthy since they will typically develop a more advanced form of the disease. It is still unclear whether rectal prolapse is purely an elastic fibre disorder, the changes that are noted may be the result of changes that occur with respect to other components of the extracellular matrix.

The remaining areas of study have focussed largely on those factors that may be involved in disease progression; changes have been noted in relation to proteolytic activity within

the pelvic connective tissues of patients with prolapse. In some cases this increased activity may have a deleterious effect as it is associated with a decrease in structural components of the extracellular matrix of some individuals with prolapse. The true ramifications of this are still as yet unclear. It is probable that upstream signalling is altered in some patients and to this end changes that have been seen with respect to TGF β in the pelvic connective tissues of patients with prolapse may be important. Indeed, those who have very little TGF β do seem to present with a more advanced form of the condition. It has been previously recorded that changes in the TGF β receptor may produce a condition which resembles Marfans, a connective tissue disorder that affects fibrillin 1. Given that changes have been seen systemically in association with dermal elastic fibres and external prolapse and TGF B in pelvic connective tissues and external prolapse. It is possible that a subgroup of patients may have not only an underlying connective tissue disorder, but also an altered response to the process of tissue injury, resulting in more rapid disease progression than would otherwise be the case.

Until now some authorities in the arena of pelvic floor surgery have doubted the existence of internal rectal prolapse and will typically attribute symptoms of internal rectal prolapse to irritable bowel syndrome (without a full diagnostic work up). It is clear from studying the pelvic connective tissues of these patients that internal rectal prolapse is associated with very similar tissue responses in terms of cellular changes to the external version of the condition and it would seem illogical to continue to regard the condition of internal rectal prolapse as a purely artificial entity. Far more important is the pressing need to study those features that serve a protective function in some patients that prevent disease progression. Myofibroblasts may be one cell type that is important

in this latter process, the lack of differentiation of the fibroblasts of the pelvic connective tissues of some patients with prolapse may not only have direct mechanical consequences but also far more wide reaching sequlae in relation to ECM component synthesis.

Further study into this complex multisystem disorder is required. The microscopic changes that have been identified with alterations in the structural organisation of the ECM may be associated with altered mechanical properties within the tissues. Since rectal prolapse is a largely mechanical disorder, understanding of the mechanical forces involved in the development and progression of the disorder is needed. Since many patients with rectal prolapse have benign joint hypermobility, these individuals should probably form the focus of a genetic study and the emerging new technique of exome analysis may allow a targeted approach to these individuals.

One of the major problems in studying this disorder is the lack of an animal model that can be used to try and identify the sequence of changes that result in the eventual development of external rectal prolapse. The studies outlined in this thesis captured events at a single time point in a heterogenous study population. By developing an animal model the ability to control confounding variables will be improved and increase the ability to focus on stages of disease development and progression.

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Appendix 1

Oxford Radcliffe Hospitals NHS
NHS Trust

HH/GR/5901

From the R & D Lead
Research & Development
Room 13, Manor House
The John Radcliffe Hospital
Headley Way, Headington
Oxford OX3 9DZ

Tel: (01865) 222147
Fax: (01865) 222648
Email: Gordon.Riddell@orh.nhs.uk

Mr Ian Lindsey
Consultant Colorectal Surgeon
John Radcliffe Hospital
Oxford

26th February 2010

Dear Mr Lindsey

Re: Investigation into collagen abnormalities and rectal prolapse

Research and Development Reference: 5901
Research Ethics Committee Reference: 09/H0603/14

Confirmation of Trust Management Approval

On behalf of the Oxford Radcliffe Hospitals NHS Trust, I am pleased to confirm Trust Management Approval and Indemnity for the above research on the basis described in the application, protocol and other supporting documents.

Conditions of Approval

The Approval is given provided that you have a substantive or honorary contract with the Trust and that you have obtained Ethical Approval. You should also comply with the conditions set out within the attached document. Please ensure that you read these conditions carefully.

Research Sponsorship

The Oxford Radcliffe Hospitals NHS accepts the role of Research Sponsor for this study in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004 and/or, the requirements of Department of Health Research Governance Guidelines.

Site Specific Assessment

This Trust Management Approval letter also incorporates site specific assessment for the Oxford Radcliffe Hospitals NHS Trust site.

Approved Documents

Document Type	Version	Date
Protocol	2	20 February 2009
Participant Information Sheet	4	07 May 2009
Consent Form	2	07 May 2009
Principal Investigator's CV	Ian Lindsey	
Investigator's CV	Edward Smyth	
Peer Review	Dr Jill Urban	16 February 2010
E-mail confirming funding	Mr Edward Smyth	22 February 2010
REC Favourable Opinion		24 June 2009
NHS REC Form		11 March 2009 (CI signature)
NHS SSI Form		12 March 2009 (PI signature)

I wish you every success with the study.

Yours sincerely,

Ms Heather House
Research & Development Lead

Copy to: Mr Edward Smyth

Enc: Conditions of Approval for Non CTIMPs

Milton Keynes Research Ethics Committee
Room 7B, PGEC
Milton Keynes Hospital Site
Standing Way
Eaglestone
Milton Keynes
MK6 5LD

01908 243750 (tele/fax)

24 June 2009

Mr Ian Lindsey
Consultant Surgeon
John Radcliffe Hospital
John Radcliffe Hospital
Headington Road
Oxford
OX3 9DU

Dear Mr Lindsey

Study Title: **Investigation into collagen abnormalities and rectal prolapse**
REC reference number: **09/H0603/14**
Protocol number: **2**

Thank you for your letter of 07 June 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
Covering Letter	E Smyth	20 April 2009	
Protocol	2	20 February 2009	
Investigator CV	I Lindsey	10 January 2009	
Application	2 IRAS	12 March 2009	
Response to Request for Further Information	E Smyth	07 June 2009	
Participant Consent Form	2	07 May 2009	
Participant Information Sheet: Rectal Prolapse and Collagen Analysis Control Group	4	07 May 2009	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments

- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0603/14	**Please quote this number on all correspondence**

Yours sincerely

Dr J Zachariah
Chair

Email: scsha.MiltonKeynesREC@nhs.net

Enclosures: "After ethical review – guidance for researchers" *SL- AR2 for other studies*

Copy to: *Ms Heather House*
Mr Edward Smyth

Appendix- 2Collagen IHC protocol from Robert Jones and Agnes Hunt Hospital, Oswestry, UK.

Provided courtesy of Prof. S. Roberts.

IHC GENERAL W collagens: METHOD FOR Mab COLLAGENS wax sections

All steps carried out at room temperature.
Dewax slides xylene 2 x 5 minutes each, re-hydrate sections in 100, 100, 90, and 70% isopropyl alcohol 2mins each

1. Pretreat sections with 0.1% hyaluronidase and 0.2% trypsin in hyaluronidase buffer at 37°C for 1 hour.
   Trypsin (Sigma) (0.2%) with 0.1% (3000u/ml approx.)
   Hyaluronidase sheep testes type V (Sigma Cat no H6254)

   Hyaluronidase buffer: 0.025 M sodium chloride (0.145gm)
   0.05 M sodium acetate (0.41gm) in distilled water pH 5.0
   (Store -20°C) for unmasking collagen epitopes in wax sections.

2. Wash with PBS 3 changes

3. Incubate with primary antibody diluted in PBS
   Anti collagen type I 1:500
   Anti collagen type III 0.25ug/ml

4. Wash with PBS 3 changes
5. Incubate with biotinylated labelled anti mouse IgG 30 minutes
   (Vectastain Elite Kit PK6102 Mouse, Vector Laboratories)
   Dilute 15µl normal serum from kit
   5µl biotinylated antibody in 1ml PBS

6. Wash with PBS 3 changes
7. Block endogenous peroxidase 0.3% hydrogen peroxide in methanol 30 min
8. Wash in PBS
9. ABC reagent from kit 30 min
   N.B. Make 30 min before needed 20µl A in 1ml PBS mix then
   add 20µl B mix well leave to stand for conjugate to form
10. Wash with PBS 3 changes
11. DAB (Diaminobenzadine) for 6 min
    add 20µl hydrogen peroxide /5ml of DAB solution add just before use and filter
12. Wash with PBS 3 changes
13. Dehydrate through series of iso-propyl alcohol 70%, 90%,100% x2
14. Clear Xylene x2
15. Mount DPX or Pertex.

Store aliquots of primary antibody at -20^{o}C. Do not freeze thaw; keep a working amount in the fridge.

Appendix 3

Glossary

Altmeirs procedure

Operation in which the rectum/ sigmoid colon is resected from the perineum.

Colonoscopy

Procedure in which the internal aspect of the colon and rectum are inspected using a flexible endoscope.

Delormes Procedure

Resection of redundant rectal mucosa from an external rectal prolapse.

Denonvilliers Fascia

Condensation of endopelvic fascia lying between the rectum and prostate gland.

Dentate line

This marks the anatomical point of separation of the internal aspect of the rectum and the anus.

Diathermy

Surgical energy device in which electrical current is converted to heat which is then used to divide tissues.

Endopelvic fascia

The fibrous connective tissue that marks the point of transition between the organs and fatty tissue of the pelvic floor and the muscles of the inner pelvis.

Foramina

Any anatomical structure that permits the passage of another structure between two separate anatomical regions.

Perineum

The perineum is the region of the external aspect of the inner pelvis and contains the anus and either vagina or scrotum. It extends from the inferior aspect of the sacrum posteriorly to the external genitalia anteriorly. It is continuous with the innermost aspect of the thigh laterally.

Peritoneal cavity

The peritoneal cavity is a region of the abdomen lined by the epithelial membrane (the peritoneum). It contains the stomach, spleen, small bowel, parts of the colon and the upper most part of the rectum. Structures may be anatomically defined as being intra peritoneal, in which case they lie wholly within the peritoneal cavity, or extra peritoneal, where they lie outside the

peritoneal cavity. Some organs such as the colon and rectum have components that are partly located in the peritoneal cavity and partly located outside it. Organs that are located entirely within the peritoneal cavity derive their blood supply from a layer of fatty tissue that runs out towards them (the mesentery).

Peritoneal reflection

The most inferior aspect of the intra peritoneal abdominal cavity marking the point of passge of abdominal viscera between the hollow part of the intra peritoneal abdomen and the fatty tissue of the pelvic floor.

Pouch of Douglas

The most inferior aspect of the peritoneal cavity lying between the uterus and rectum

Proctogram (c.f. defecating proctogram)

A radiological study designed to allow a detailed study of the rectum. Radio-opaque contrast is introduced into the rectum and real time cine radiography or MRI scanning is used to gain dynamic images of rectal movement.

Inguinal ring (inguinal canal)

This anatomical region is located between the anterior superior iliac spine and the pubic tubercle. It is roughly located one hands bredth laterally from the symphysis pubis. It lies posterior to the external oblique aponeurosis muscle of the anterior abdominal wall. It transmits the testicular cord structures in males and the uterine round ligament in females.

Sacral nerve stimulation

Operation in which the sacral nerves are artificially stimulated to improve the function of the pelvic nerves and treat faecal incontinence or bowel dysfunction.

Sacral promontory

The sacral promontory marks the point of transition between the lumbar spine and the sacral bones. The human spine is not straight and therefore the uppermost part of the sacrum projects anteriorly forming a bony structure, not dissimilar to a table, this is the sacral promontory.

Thresholding

When image analysis software is used to process an image the structure of interest can be extracted from the sample according to its colour and density. This is achieved by adjusting the displayed image such that only certain structures are displayed, this process is termed thresholding.

Ventral mesh rectopexy

Abdominal procedure designed to correct both internal and external rectal prolapse by mobilisation of the rectum and repair using a prosthetic mesh.

Waldeyers fascia

Condensation of endopelvic fascia supporting the posterior pelvic rectum.

Appendix 4

List of suppliers

AdB Serotec Ltd

Endeavour House

Kidlington

Oxford

OX5 1GE

VWR International Ltd

Hunter Boulevard

Lutterworth

LE17 4XN

ABCAM

330 Cambridge Science Park

Cambridge

CB4 0FL

Vector Laboratories

3 Accent Park

Peterborough

PE2 6XS

Raymond Lamb

c/o Thermo Scientific Ltd

Tudor Road

Manor Park

Runcorn

Cheshire

WA7 1TA

Brunel Microscopy Ltd

Unit 2, Vincents Road

Bumpers Farm Industrial Estate

Chippenham

Wiltshire

SN14 6NQ

DAKO UK Ltd

Cambridge House

St. Thomas Place

Ely

Cambridgeshire

CB7 4EX