The Aetiology and Pathogenesis of Rectal Prolapse



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Abstract

It is still an enigma that some patients develop rectal prolapse whilst others with similar risk factors do not. Biomechanical assessment of the skin may provide further insight into the aetiology of this complex condition. Components of connective tissue other than collagen have been found to be involved in patients with rectal prolapse. Elastin fibres are an abundant and integral part of many extracellular matrices and are especially critical for providing the property of elastic recoil to tissues. The significance of elastin fibres is clearly reflected by the numerous human conditions in which a skin phenotype occurs as a result of elastin fibre abnormalities. The organization of elastic fibres differs between controls and subsets of patients with rectal prolapse, and their importance for maintaining the structural and functional integrity of the pelvic floor has been demonstrated in transgenic mice, with animals which have a null mutation in fibulin-5 (Fbln5¡/¡) developing prolapse. This study aimed to compare fibulin-5 expression in the skin of patients with and without rectal prolapse. Between January 2013 and February 2014, skin specimens were obtained during surgery from 20 patients with rectal prolapse and from 21 without prolapse undergoing surgery for other indications. Fibroblasts from the skin were cultured and the level of fibulin-5 expression was determined on cultured fibroblasts, isolated from these specimens by quantitative real-time polymerase chain reaction. Immunohistochemistry was performed on fixed tissue specimens to assess fibulin-5 expression. Orcein staining measured expression levels of elastin in the skin, and Image J. Tensile tests were performed using the Zwick Roell device, with custom ceramic clamps. For statistical analysis, Student's t test was used. Fibulin-5 mRNA expression and fibulin-5 staining intensity were significantly lower in young male patients with rectal prolapse compared with age matched controls [fibulin-5 mean _

SD mRNA relative units, 1.1 _ 0.41 vs 0.53 _ 0.22, P = 0.001; intensity score, median (range), 2 (0–3) vs 1 (0–3), P = 0.05]. There were no significant differences in the expression of fibulin-5 in women with rectal prolapse compared with controls. Histological analysis of prolapse vs control showed percentage dermal elastin fibres of 9 vs 5.8 % (p=0.001) in males and 6.5 vs 5.3 % (p=0.05) in females. Patients with more severe prolapse (external) had a significantly (p=0.05) higher percentage dermal elastin fibres 6.9 vs 6.1 % than internal prolapse. Young's modulus of patients with prolapse was lower in males (3.3 vs 2.8, p=0.05) and females (3.1 vs 2.7, p=0.05). Patients with prolapse have a higher concentration of elastin fibres in the skin, and these differences are quantitatively demonstrated through mechanical testing. This suggests that the aetiology may be a result of a dysfunction of elastin fibre assembly. Fibulin-5 may be implicated in the aetiology of rectal prolapse in a subgroup of young male patients.

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I dedicate this thesis to my late grandmother Hiraben Narottam Joshi (“Ba”) and my two daughters Rhia and Bani aged 7 and 3 who were named in memory of her.

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STATEMENT OF ORIGINALITY

All the investigations and results described in this thesis were undertaken by the candidate, in the University of Oxford Departments of Physiology, Anatomy, Genetics and Zoology. However, the histology was performed with assistance from Richard Stillion in the Dunn School of Pathology.

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PUBLICATIONS ARISING FROM THIS THESIS

Papers

Joshi HM, Woods AK, Smyth E, Gosselink MP, Cunningham C, Lindsey I, Urban J, Jones OM, Vollrath F. Histological and mechanical differences in the skin of patients with rectal prolapse.

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Joshi HM, Gosselink MP, Smyth EA, Hompes R, Cunningham C, Lindsey I, Urban J, Jones OM. Expression of fibulin-5 in the skin of patients with rectal prolapse.

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Abstracts

Joshi HM, Gosselink MP, Smyth EA, Jones OM
Fibulin-5 expression in the skin of patients with rectal prolapse
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Joshi HM, Woods AK, Smyth E, Gosselink MP, Cunningham C, Lindsey I, Urban J, Jones OM, Vollrath F. Biomechanical properties of pelvic connective tissue. *Colorectal Disease* 2014 (Suppl. 2), 45

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LIST OF ABBREVIATIONS

AR	autosomal recessive
ATFP	arcus tendineus fascial pelvis
BGN	biglycan
BJH	benign joint hypermobility
CCA	congenital contractural arachnodactyly
DCN	decorin
DMA	dynamic mechanical analysis
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
ECM	extra cellular matrix
FBS	foetal bovine serum
FBN	fibulin
FMOD	fibromodulin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
LTBP	latent TGF-β-binding proteins
LOX	lysyl oxidase
MAGP	microfibril-associated glycoproteins
MFS	Marfan syndrome
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
N	Newton
PCR	polymerase chain reaction
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
PBS	phosphate buffered saline
POD	pelvic organ dysfunction
POP	pelvic organ prolapse
ROX	X-rhodamine
RNA	ribonucleic acid
USL	utero-sacral ligament

LIST OF REAGENTS

Acetone	Invitrogen, Paisley, UK
Antibiotic/Antimycotic	Invitrogen, Paisley, UK
Avidin-biotin complex	Thermo Fisher Scientific , MA USA
10% Isopropanol	Sigma-Aldrich Ltd, Dorset UK
Loading buffer 5x	Invitrogen, Paisley, UK
Dulbecco modified Eagle Medium DMEM	Invitrogen, Paisley, UK
Dulbeccos phosphate buffered saline (1x) PBS	Invitrogen, Paisley, UK
Eosin–Y–alcoholic solution	Invitrogen, Paisley, UK
Ethanol EtOH	Invitrogen, Paisley, UK
Fetal bovine serum	Invitrogen, Paisley, UK
Forene (isoflurane)	Invitrogen, Paisley, UK
Glycerol	Sigma-Aldrich Ltd, Dorset UK
Harris Haemotoxylin	Leica Microsystems Ltd, UK
Methanol	VWR, Darmstadt, GermanyMonoclonal mouse Anti-Fibrillin1 Abcam, Cambridge, MA, USA
Monoclonal mouse Anti-Fibulin-5	Abcam, Cambridge, MA, USA
Nanoscript2 kit	Primerdesign Ltd, York, UK
Normal donkey serum	Sigma, Saint Louis, USA
Normal mouse serum	Sigma-Aldrich Ltd, Dorset UK
Paraffin	Sigma-Aldrich Ltd, Dorset UK
Paraformaldehyde 4 %	Sigma-Aldrich Ltd, Dorset UK
Penicillin/Streptomycin P/S	Invitrogen, Paisley, UK
Orcein	Leica Microsystems Ltd, UK
RNeasy Micro kit	Qiagen, Hilden Germany
RNase free DNase I	Qiagen, Hilden, Germany
Sodium chloride solution NaCl	Invitrogen, Paisley, UK
Tris–Hydrochloride	Invitrogen, Paisley, UK
Trypsin [10 mg/ml]	Invitrogen, Karlsruhe, GER
Trypan blue	Sigma-Aldrich Ltd, Dorset UK
Tween 20	Sigma-Aldrich Ltd, Dorset UK
Vectashield mounting media	Vector Lab, Peterborough, UK

CHAPTER 1

INTRODUCTION

1.0 Introduction

Pelvic floor disorders constitute an increasing problem as the population ages. The literature and experimental work has concentrated almost exclusively on the pathogenesis of pelvic floor disorders such as uterine prolapse and urinary incontinence and in the development of abdominal hernia (Fachinelli et al. 2011; Bakas et al. 2004). Little basic science research has been undertaken evaluating the connective tissue changes in patients with rectal prolapse and thus no literature is available to review in this regard. To date, apart from our group in Oxford (Smyth E 2012) no studies have directly assessed the changes in the connective tissues of patients suffering from rectal prolapse. Given the prevalence of the disorder and the paucity of relevant basic science in relation to its pathogenesis this would seem an important area for research and is the area of this thesis.

1.1 Anatomy of the pelvic floor and rectum in man and other species

The rectum is supported by a highly interdependent physiologically complex system comprised of striated muscle, smooth muscle, and connective tissue (Mahadevan 2011). This complex is often referred to as the pelvic floor. The striated muscle component, referred to as the levator ani muscle complex comprises the inferior most portion and consists of the pubococcygeous, ileococcygeous and coccygeous muscles. Tonic contraction of the levator ani provides support to pelvic organs by acting as a shelf upon which they rest with anatomical interruptions to afford passage of the urethra, vagina and rectum. Current dogma generally assumes that both the levator ani

and connective tissues are necessary for normal rectal support in the human (Bennett 2008; Mahadevan 2011). However, one of the main differences between humans and every other animal is that humans are bipedal. In addition, humans have lost the need for a tail. Thus, as a consequence of walking upright, the human anatomy appears to have adapted by recruiting the striated muscle, which is important for tail function in so many other species, to assist in resisting the downward forces resulting from a combination of abdominal pressure and gravity (Hollinshead 1962; Nano et al. 2000). Thus, a load sharing relationship has developed between the connective tissue and striated muscle making both critical to the maintenance of normal rectal support in humans.

Many mammals appear to have analogous connective tissue support to the human. However, the major anatomical difference between humans and other species appears to be the degree to which the levator ani muscles contribute to rectal support. In animals with more bipedal behavior (e.g. Rhesus Macaques), the levator ani muscles appear to have more of a role in rectal support than in quadrupeds (e.g. rat), for which the primary function of the levator muscles is movement of the tail (Stoker 2009). Thus, when comparing animal studies, it is important to recognize these anatomical differences and whether the primary support to the pelvic organs is provided primarily by connective tissue or a combination of both muscle and connective tissue. Depending on the research question under investigation, the rat, despite its anatomical differences, may indeed be a better choice because it offers the opportunity to isolate the contribution of the connective tissue component. This emphasizes the importance of clearly defining the research question for our study, when considering pelvic anatomy, histological appearance, biochemical constituents, and mechanics.

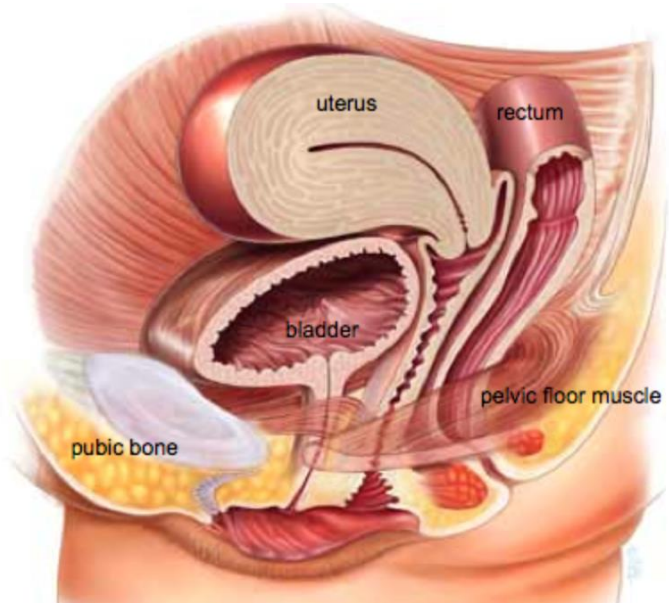


Figure 1.0a Normal anatomy of the pelvic floor (taken from https://femalepelvicfloorcentral.wordpress.com)

1.2 Rectal prolapse

Supports for pelvic organs include the bony pelvis, pelvic floor muscles and fascial supports. The endopelvic fascia is composed of collagen, elastin and smooth muscle. This pelvic supportive tissue contains blood vessels, nerves and fibrous connective tissue and therefore functions as a combined neurovascular and supportive structure. Pelvic organ prolapse is said to be due to mechanical, neurological and connective tissue factors.

Rectal prolapse is characterised by external protrusion of the rectum from its usual anatomical position with associated pelvic floor dysfunction. A prodromal phase of internal prolapse is also recognised (Jones et al. 2011) . Internal prolapse presents insidiously manifesting as symptoms ranging from obstructed defecation through to

faecal incontinence (Wijffels et al. 2010; Harmston et al. 2011). It is a common disorder and 1 in 1000 patients will undergo surgery for the condition in the United Kingdom each year (Keane et al. 1997).



Figure 1.0b: Surgical photograph of a clinical rectal prolapse (taken from www.emedicne.medscape.com)

The aetiology of the condition is not well defined. The traditional view is that it occurs predominantly in multiparous ageing women through a pathway of direct pelvic floor injury resulting from parturition(Harmston & Jones 2011). However, about 20-30% of patients are either nulliparous or male, 2 subgroups in whom this explanation is inadequate. Understanding the cause of this condition is critical to improving treatment and prevention; but clarifying causation is complex because of their multifactorial nature. Factors such as instrumented vaginal delivery, heavy lifting, smoking, rapid decline in tissue strength, damaged levator ani muscle, and nerve injury are cited as increasing the likelihood that a patient may develop prolapse

or experience recurrence of pelvic floor dysfunctions after surgery. Rather than a single factor, it is more probable that combinations of anatomical, physiological, genetic, lifestyle, and reproductive factors interact throughout a life span to contribute to prolapse. DeLancey et al. compared levator ani defects in MRI images from women with and without prolapse and found that 55% of women with prolapse had measurable MRI defects (DeLancey et al. 2003). Pierce et al. challenged the levator injury model in a well-controlled animal study in which the levator ani nerve of nulliparous squirrel monkeys was ablated via an excisional biopsy (Pierce et al. 2007). Interestingly, although all of the monkeys with ablated nerves had evidence of marked levator atrophy by MRI, none developed pelvic organ descent; thus, challenging the central role of levator injury in the pathogenesis of prolapse.

The injury, recovery, and disease mechanisms that affect each type of tissue and structure must be individually considered. For example, during birth, the striated muscle could sustain irrecoverable injury because of the rupture of the muscle from excess stretching, denervation from nerve compression or elongation or ischemic injury because of compartment syndrome. Connective tissue change with advancing age could be due to gradual stretching due to mechanical deterioration, or it could rupture during a single episode of structural overload (Batista et al. 2011). Each type of tissue has specific types of injury; specifying the proposed mechanism of impairment can focus research on forward progress.

1.2.1 Predisposing factors

Understanding the cause of this condition is critical to improving treatment and prevention; but clarifying causation is complex because of their multifactorial nature. It is likely to be a combination of anatomical, physiological, genetic, lifestyle, and reproductive factors that interact throughout a life span to contribute to prolapse. Having a framework that integrates how different factors interact to result in the development of rectal prolapse can facilitate understanding these complex causal questions,

There are normal variations in initial growth and development of all parts of the body. Variation in height is perhaps the most familiar. Pelvic floor development, like height, is influenced by factors such as an individual's genetic code, nutrition, and environment. An individual who develops excellent pelvic floor function may never have sufficient deterioration to develop rectal prolapse symptoms throughout life span, despite inciting or lifestyle events. However, another individual whose growth and development are less robust may become symptomatic during their lifetime solely on the basis of normal age-related decline. Conceptually then, the role of genetics must be considered in the analysis of rectal prolapse causation.

The pathophysiology that affects each type of tissue and structure must be individually considered. For example, during birth, the striated muscle could become damaged from excess stretching, denervation from nerve compression ischaemic injury due to compartment syndrome (Rahn et al. 2008). Connective tissue change

with advancing age could be due to gradual stretching due to mechanical deterioration, or it could rupture during a single episode of structural overload.

The normal decline of the pelvic floor across the life span may be influenced by other factors. Chronic constipation, obesity, immunosuppression with steroids and diabetes are influential as are occupations that involve heavy lifting or conditions that elicit heavy coughing, such as chronic bronchitis, may result in a type of repetitive motion trauma that challenges the pelvic floor. Any of these factors, or combination of factors, can accelerate loss of pelvic floor function over the course of a life span (Nygaard 2008)

1.3 Structure and organization of rectal supportive connective tissue

The possible relationship between pelvic organ prolapse and connective tissue factors has been indirectly implied by the repeated association of clinically significant prolapse and joint hypermobility in women (Bø et al. 1994; Zarate et al. 2010). Although there is a significant amount of published literature on the management of pelvic organ prolapse (Olsen et al. 1997; Nygaard 2008; McNevin 2010), there is comparatively little on the elucidation of biochemical and biomechanical properties of pelvic organ supports and their roles in maintaining normal anatomy and prolapse. There is also increasing use of prosthetic materials such as mesh in the management of vaginal prolapse (O'Grady et al. 2000). Most of the meshes used, however, are designed for use elsewhere in the body, such as abdominal hernia.

The supporting structures of the pelvic floor are composed mainly of connective tissue (extracellular matrix – ECM) containing fibrous elements (collagen and elastic fibrs), proteoglycans and a smaller amount of non-collagenous glycoproteins, hyaluronan and proteoglycans (Kerkhof et al. 2009). In addition, the supportive connective tissues contain a significant amount of smooth muscle. An understanding of connective tissue remodeling is of interest because it directly affects the mechanical integrity of the rectum and its supportive tissues (Lin et al. 2007).

1.3.1 Collagens

The extracellular matrix of the pelvic floor is comprised predominantly of collagens, Collagen I confers the properties of tensile strength to tissues (Lammers et al. 2012). Collagens III and V, are usually associate with collagen I and help regulate the diameters of collagen fibrils. An increase in collagens III and V is associated with decreases in the mechanical integrity of connective tissue (Goh 2003; Jelovsek et al. 2007). Moalli et al. showed that premenopausal women on hormones had a higher ratio of collagens (I/III + V) in their arcus tendineus fascia pelvis than menopausal women not on hormones (Moalli et al. 2006; Lim et al. 2014) Thus, it is likely that hormones play a role in regulating the mechanical properties of the supportive connective tissues.

In the rectum, collagen comprises the primary structural component (84%) with an intermediate amount of elastin (13%). Analyses of the rectal attachments in both rodents and primates have shown that they also consist primarily of collagen with a smaller amount of elastin and smooth muscle (Lim et al. 2014). Elastin provides the

tissue with resilience (Kim et al. 1999) which is crucial to the function of dynamic connective tissues such as arteries, lung, skin and most likely the pelvic floor including the connective tissue attachments between the vagina and the pelvic sidewall (Lin et al. 2007; Mosier et al. 2010). The elastin fibres are designed to maintain elastic function throughout life apart from in the reproductive tract in which they are degraded and re-synthesized following vaginal delivery. Collagen and elastin degrading enzymes, particularly, the matrix metalloproteinases (MMP) have been found to be up-regulated in the vagina of women with uterine prolapse (Dviri et al. 2011; Phillips et al. 2006; Manzetti et al. 2003). In addition, mice with null mutations in genes involved in elastin synthesis develop spontaneous prolapse (Klutke J, Ji Q, Campeau J, Starcher B, Felix JC, Stanczyk FZ 2008; Sultan 2009).

1.3.2 Proteoglycans

Decorin (DCN), fibromodulin (FMOD), biglycan (BGN), luminican, and chondroadherin are small proteoglycans, which provide tissues with adherence. They interact with collagen and elastin fibres to form networks of surrounding fibres that resist compression by trapping water molecules (Reed & Iozzo 2002). They also bind growth factors, influencing cell proliferation, migration and adhesion, and create viscoelastic and turgor pressures (Kishioka et al. 2008). Larger proteoglycans like aggregan, versican and perlecan, are responsible for maintaining tissue hydration and contribute to the overall structural scaffolding in the ECM (Kiani et al. 2002). The significance of ECM proteoglycans has not received much attention for rectal prolapse, but very well may play an important role in ECM remodeling (Söderberg et al. 2009; Trabucco et al. 2007).

1.3.3 Elastin and Elastic Tissues

1.3.3.1 Elastin.

Elastin is a component of ECM and has a role in conferring properties of elasticity and recoil to tissues in all organs of the body. Disrupted elastin metabolism can lead to vascular and respiratory pathology. Cross-linkages between elastin and collagen fibres give tissue strength and are formed by the enzyme lysyl oxidase (LOX). Elastin is synthesized and broken down in the female genital tract and facilitates childbirth and recoil post labour. An analysis of uterosacral ligaments in women with POP found that elastic fibres were undetectable on immunofluorescent staining or sometimes fragmented in women with POP (Gabriel et al. 2006; Strinic et al. 2010). Another case-control study showed that elastin fibres were smaller and less expressed in vaginal tissue of women with POP (Klutke J, Ji Q, Campeau J, Starcher B, Felix JC, Stanczyk FZ 2008). A lower elastin content and decreased mRNA expression of LOX was found in uterosacral ligaments of women with POP compared with normal controls (Alperin et al. 2008).

Elastin content is also important in stress urinary incontinence, as lower and more degraded elastin complexes have been noted in the periurethral tissues of women with ‘hypotonic’ stress urinary incontinence (Goepel & Thomssen 2006). Damage to elastin fibres can stimulate not only its own remodeling, but that of adjacent collagen fibres as well. Certain MMPs are capable of degrading elastin (Budatha et al. 2011). Women with stress urinary incontinence have been shown to have a threefold increase in systemic elastase activity (B. Chen et al. 2004), possibly contributing to collagen

degradation. This imbalance between elastase proteolytic activity and its inhibitor relates back to MMP-1 and TIMP-1 activity in the collagen metabolism, as incontinent women may have higher elastin degradation attributable to lower elastase inhibition (Budatha et al. 2011). Lower levels of fibrillin-1, an essential elastin scaffold, have been shown in periurethral specimens of women with stress urinary incontinence, which may lead to impaired deposition of elastin (Chen et al. 2002). Overall, a highly active elastin remodeling state may result in weakened ultrastructure, ultimately causing stress urinary incontinence. Studies investigating elastin content and metabolism is varied, but shows a trend for a decreased level of elastin in pelvic tissues using methods such as immunohistochemistry, Western immunoblotting and real-time PCR.

1.3.3.2 Fibrillins

Fibrillins-1 and 2 are major structural components of the extracellular microfibrils with average diameter of 10 nm. Fibrillin-containing microfibrils are distributed in a wide variety of tissues and organs, either associated with elastin in the elastic fibres or as elastin-free bundles. Fibrillins are cysteine-rich glycoproteins made of structural motifs that are also found in other microfibril-interacting molecules, namely fibulins-1 and 2 and latent TGF-β-binding proteins-1 and 2 (LTBP-1 and LTBP-2). The bulk of fibrillin-1 synthesis correlates with late morphogenesis, whereas fibrillin-2 production peaks earlier and before overt elastogenesis (Zhang et al. 1995). Fibrillin-1 mutations cause the pleiotropic manifestations of Marfan syndrome (MFS); fibrillin-2

abnormalities result in the skeletal abnormalities of congenital contractural arachnodactyly (CCA) 4 and 5.

1.3.4 Fibulin-5

Fibulin-5 is a matricellular protein, contributing to the structural development of elastogenic tissues, as well as mediating various cellular functions required for the maintenance of tissue homeostasis (Hu et al. 2006). A diverse cellular effect of fibulin-5, in a cell-type specific and context-dependent manner, suggests that there may be multiple fibulin-5-interacting proteins at the cell surface, including integrins (Northington 2011). It remains to be determined how fibulin-5 mediates or antagonizes cellular signaling at the cell surface, and whether modulation of fibulin-5 has the potential for the therapy of pelvic floor pathologies (Rahn et al. 2009).

1.3.4.1 Fibulin-5: an integrin-binding member of the Class II fibulin subfamily

Fibulins are characterized by tandem repeats of calcium-binding EGF (cbEGF)-like motifs and a globular C-terminal fibulin module (Northington 2011). Fibulin-5 was first identified in search of genes involved in phenotypic modulation of vascular smooth muscle cells and in cardiovascular development (Nakamura et al. 2002).

Fibulin-5 was shown to be a 66-kDa glycoprotein containing six calcium-binding EGF-like (cbEGF) motifs, which are believed to provide stability and facilitate

protein interaction (Hu et al. 2006). Fibulin-5 also contains a RGD motif involved in the binding to a subset of cell-surface integrins. The RGD sequence is present in various matricellular and ECM proteins, including fibronectin, vitronectin, osteopontin and thrombospondins, and is recognized by heteromeric integrin receptors to participate in cellular functions (Yanagisawa et al. 2009).

Nakamura et al identified the biological function of fibulin -5 with the generation of *Fbln5* knockout mice in which systemic elastic fibre defects were revealed (Nakamura et al. 2002; Zhao & Zhou 2012). *Fbln5*-null mice survive to adulthood but progressively develop severe elastinopathy, including loose skin, tortuous aorta, emphysematous lung, and genital prolapse. These observations established the first animal model for congenital elastic fibre defects.

1.3.4.2 Fibulin-5 and elastic fibre development

Elastic fibres are composed of an amorphous-appearing elastin core and a peripheral mantle of 10-nm fibrillin-containing microfibrils. In addition to elastin, fibrillin-1, and fibrillin-2, more than 30 microfibril- and elastin-associated proteins, have been identified, including fibulins, microfibril-associated glycoproteins MAGP-1 and MAGP-2, and latent TGF-β binding proteins-1 through -4 (LTBPs) (Kielty et al. 2002; Kielty 2006). Elastogenesis occurs through a series of highly regulated steps that involve secretion of the tropoelastin monomer, self-aggregation of tropoelastin, called coacervation, correct assembly and cross-linking of tropoelastin, and final organization of the insoluble elastin into functional fibres (Hu et al. 2006). *Fbln5* expression coincides with *elastin* expression in the aorta. During this time, the

secreted elastin is cross-linked and organized into insoluble elastin sheets or laminae (Zanetti et al. 2004).

Recently, double knockout mice for the genes encoding fibrillin-*1* (*Fbn1*) and f*ibulin-5* exhibited a severe disruption of elastic fibres and a marked dysregulation of TGF-β-mediated signals (Wachi et al. 2007) (Drewes et al. 2007). These observations, together with biochemical and ultrastructural studies, have led to the suggestion that an interaction between microfibril proteins and tropoelastin may be critical for proper elastic fibre assembly.

1.3.4.3 Role of fibulin-5 in elastic fibre assembly

A dose-dependent, direct interaction between fibulin-5 and tropoelastin was demonstrated by solid-phase binding assays, and co-localization of fibulin-5 and elastin fibres was revealed at an electron microscopic level, providing a basis for the molecular function of fibulin-5 (Yanagisawa et al. 2002). Choi et al. showed by using electron microscopy that the average size of elastin aggregates was increased in the skin of *Fbln5*-null mice, compared with wild-type mice (Zheng et al. 2006). Taken together, these findings show that fibulin-5 functions at a formation and maturation step of coacervation to 1) control coacervation efficiency, and 2) to regulate the size of self-aggregates to achieve optimal cross-linking of tropoelastin during elastic assembly (Kuang et al. 2003).

It was also demonstrated that fibulin-5 binds to fibrillin-1, a major component of microfibrils (El-Hallous et al. 2007; Freeman et al. 2005). Fibulin-5 co-localizes with microfibrils in elastogenic cells, as well as in non-elastogenic cells, engineered to

overexpress tropoelastin and fibulin-5 (Hirai et al. 2007; Nonaka et al. 2009; Zheng et al. 2007). Consistent with these observations, a significant alteration of fibulin-5 expression was observed in tight skin mice, in which a *Fbn1* mutation caused abnormally tight skin, dermal fibrosis, and impaired elastogenesis (Lemaire et al. 2004). Furthermore, it was shown that knocking down *Fbn1* in human skin fibroblasts abrogates the formation of elastic fibres and decreases fibulin-5 immunoreactivity.

Fibulin-5 also binds cross-linking enzymes, including lysyl oxidase like (Loxl)-1, -2, and -4 (Hirai et al. 2007; Liu et al. 2004).

1.3.4.4 Mutation of fibulin-5 in human elastic fibre disease

Human genetic studies have identified two homozygous missense mutations of *FBLN5* (p. S227P) and (p. C217R) in autosomal recessive (AR) cutis laxa families (Claus et al. 2008; Elahi et al. 2006; Loeys et al. 2002). The first mutation was reported in two families of ethnically different groups and the disease manifested as a severe form of cutis laxa with internal organ involvement (AR cutis laxa type I). Biochemically, both substitutions of fibulin-5 (S227P and C217R) were shown to decrease binding to tropoelastin. The S227P mutation further revealed a significant decrease in synthesis and secretion of the mutant protein and impaired association with fibrillin-1 (Hu et al. 2006). A heterozygous tandem duplication of fibulin-5 was also reported in a patient with a mild form of cutis laxa, suggesting that the large mutant protein might act in a dominant negative fashion (Markova et al. 2003).

1.3.4.5 Alteration of fibulin-5 in pathological conditions involving elastic fibres

Fbln5-null mice were shown to develop pelvic organ prolapse and have served as one of the first animal models for this condition (Drewes et al. 2007). By 6 months of age, 92% of *Fbln5*-null females developed vaginal prolapse with severe disruptions in elastic fibre formation. Biomechanical studies using vagina tissue from pregnant and

non-pregnant females from wild-type and *Fbln5*-null mice revealed that the *Fbln5*-null vagina with prolapse was similar to that of pregnant wild-type females, exhibiting a significant decrease in vaginal stiffness (Rahn et al. 2008). Subsequently, it was shown in the uterosacral ligaments of patients with pelvic organ prolapse (POP) that expression of fibulin-5 was significantly decreased (Jung et al. 2009). Additionally, fibulin-5 mRNA was decreased in para-urethral biopsies obtained from pelvic organ prolapse women (Söderberg et al. 2009), underscoring defective elastic fibres as an underlying cause of pelvic organ prolapse.

1.4 Matrix Metalloproteinases MMPs

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. The MMPs belong to a larger family of proteases known as the metzincin superfamily (Pardo & Selman 2005). These enzymes are capable of degrading extracellular matrix proteins, but also can process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (Zong et al.

2007). MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense (Pardo & Selman 2005).

1.4.1 Structure

The MMPs have a common domain structure. The three common domains are the pro-peptide, the catalytic domain, and the haemopexin-like C-terminal domain, which is linked to the catalytic domain by a flexible hinge region (Pardo & Selman 2005)

1.4.2 Classification

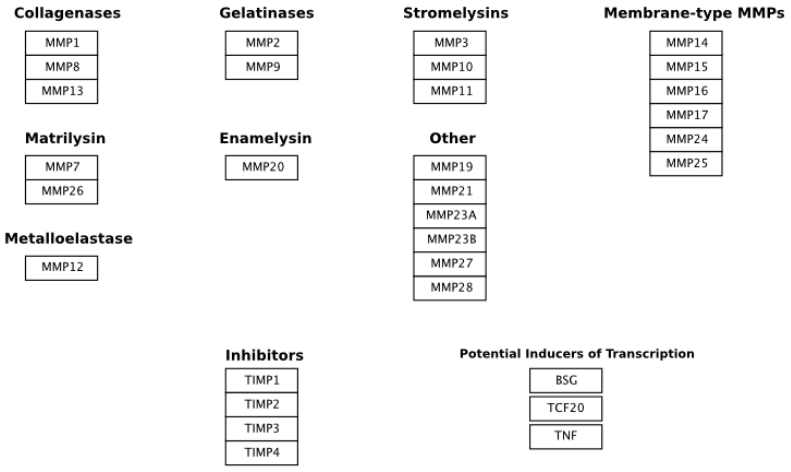


Figure 1.0 Classification of MMPs (Pardo & Selman 2005)

The most commonly used groupings (by researchers in MMP biology) are based partly on historical assessment of the substrate specificity of the MMP and partly on

the cellular localization of the MMP. These groups are the collagenases, the gelatinases, the stromelysins, and the membrane-type MMPs (MT-MMPs).

1.4.3 Genes

Gene	**Name**	**Location**
MMP 1	Interstitial collagenase	secreted
MMP 9	Gelatinase-B, 92 kDa gelatinase	secreted

Table 1.0 Gene of interest

The main substrates of the gelatinases are type IV collagen and gelatin. These enzymes are distinguished by the presence of an additional domain inserted into the catalytic domain. This gelatin-binding region is positioned immediately before the zinc-binding motif, and forms a separate folding unit that does not disrupt the structure of the catalytic domain. The gelatinases are No. 2 and No. 9.

1.4.4 Function

Throughout life, connective tissue macromolecules are both synthesized by the resident cells of the rectal support, and degraded by proteases (particularly the MMPs) also produced by these cells. In healthy adult tissues, the rates of synthesis and

breakdown are in balance so that the composition of the tissues remains constant, but under pathological conditions, breakdown overtakes synthesis and the tissues degrade.

The MMPs play an important role in tissue remodeling. MMP-9 is important in metastasis and MMP-1 is thought to be important in rheumatoid arthritis and osteoarthritis (Ham et al. 2013).

1.4.5 Activation

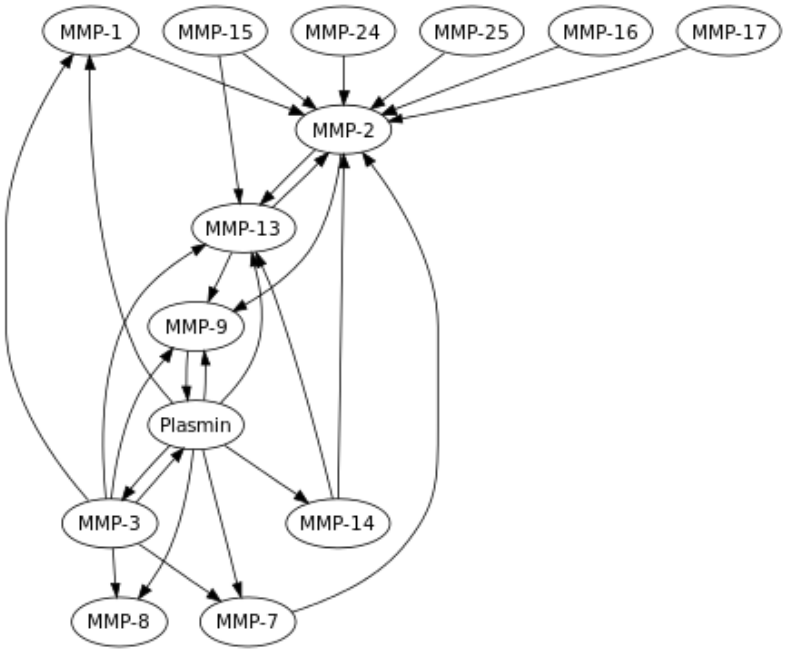


Figure 2: Activation of MMP (Trexler et al. 2003)

The gelatinases are secreted in a zymogenic form (pro-MMP) that is activated in the extracellular space and therefore zymogen activation is an important mechanism of regulation of gelatinase activity. Since several major targets of gelatinase activity are located directly on the cell surface (Werb 1997; Rafii et al. 2002), specific

mechanisms were developed by cells to promote the association and activation of the gelatinases on the cell surface.

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1.4.6 Inhibitors

MMP activity is further inhibited by TIMPS (specific endogenous tissue inhibitor of metalloproteinases), which comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Määttä et al. 2007). Disregulation of the balance between MMPs and TIMPs is a characteristic of acute and chronic cardiovascular diseases (Antoniou et al. 2011) but the role of TIMPs has not been studied in pelvic floor disorders.

1.4 7 MMPs and connective tissue degradation.

While MMPs play an important role in tissue remodeling they are also involved in tissue breakdown. MMP-9 is important in metastasis and MMP-1 is thought to be important in rheumatoid arthritis and osteoarthritis (Ham et al. 2013). Excess MMPs degrade the structural proteins of the aortic wall (Murphy & Nagase 2009). Interstitial and neutrophil collagenases (MMP-1, MMP-8, MMP-13) cleave fibrillar collagens with the resulting peptides degraded by the gelatinases (MMP-2 and MMP-9).

MMP activity has been studied in some pelvic floor disorders. Kerkoff et al. studied the vaginal-epithelial tissue of premenopausal women with pelvic organ prolapse and found increased MMP-2 and MMP-9 activity indicating increased turnover (Kerkhof et al. 2009). Also, studies have examined the relationship between collagen/elastin

degradation and pelvic floor dysfunction. There have been consistent results showing the impact of higher MMP activity in the development of stress urinary incontinence and pelvic organ prolapse, although studies have used different methodologies (Chen et al. 2002; Lohi et al. 2001) . The contribution of the TIMPs to this process remains to be established.

1.5 Inherited predisposition.

In some patients, there may be an inherited predisposition to rectal prolapse. A significant proportion of patients with functional gastrointestinal disorders and hernias have been noted to suffer from benign joint hypermobility syndrome (Arunkalaivanan et al. 2009; Jha et al. 2007). Inherited disorders of the extracellular matrix appear to be involved in some cases. In children, thus rectal prolapse has been associated with Ehlers-Danlos Syndrome (Aydeniz et al. 2010). This syndrome consists of a heterogeneous group of clinical features caused by underlying defects in the collagen type I, III and V gene as well as defects in proteins important for the post-translational modifications and processing of these collagens (Callewaert et al. 2008) It is known that female patients with EDS have a higher risk of developing pelvic organ prolapse (Carley & Schaffer 2000). Besides, both male and female patients with one of the different subtypes of EDS reported a higher prevalence of joint hypermobility; arterial rupture; rupture of diaphragm; atrophic scarring; varicose veins; cardiac valvular disease, such as cardiac valve prolapse; rectal prolapse and inguinal, umbilical or other hernias (Callewaert et al. 2008). Other investigators have focused on these different diseases in the general population and their relation to collagen. They found

changes in collagen quantity or composition of the different collagen subtypes in for example patients with pelvic organ prolapse (Kerkhof et al. 2009; Jackson et al. 1996), inguinal hernia (Meyer et al. 2007; Ozdogan et al. 2006), varicose veins (Haviarova et al. 2008), arterial aneurysm (Carmo et al. 2002) and joint hypermobility (Malfait et al. 2006). This suggests a common pathophysiologic pathway for both prolapse and the above-mentioned disorders. Work, focusing on collagen defects as an aetiological factor, has thus been undertaken on patients with these disorders, Changes in collagen fibre cross links and disruptions of collagen 1 and 3 ratios have been noted in nulliparous patients with stress urinary incontinence (Bø et al. 1994). Results have been conflicting and other groups have not noticed similar changes (Reilly et al. 2008).

Elastic tissue mutations may also predispose to pelvic floor disorders. Elastic fibres are component of the extracellular matrix comprising an elastin core surrounded by a mantle of fibrillin-rich microfibrils. Elastin fibres confer tissues with properties of elasticity and resilience (Fachinelli et al. 2011) . Dysfunction in the fibrillar proteins occurs in Marfan syndrome, leading to lax connective tissues and a predisposition to disorders such as aneurysms(Neptune et al. 2003). In animal models dysfunction of fibrillar proteins has been associated with the development of pelvic organ prolapse (X. Liu et al. 2006; Drewes et al. 2007; McLaughlin et al. 2006). The colonic mucosa of rectal prolapse samples in humans is associated with increased deposition of elastic fibres (Rahn et al. 2008; Rahn et al. 2009).

Turnover of tissue proteins (collagen, elastin and other components of the extracellular matrix) occurs by the tightly regulated activity of a class of enzymes,

matrix metalloproteinase, and their inhibitors. In uterine prolapse and abdominal wall hernias the activities of this class of enzymes are up regulated (Aydeniz et al. 2010).(Olsen et al. 1997; Keane et al. 1997; Y. Chen et al. 2004; Rahn et al. 2008; Casanova et al. 2009) These studies show conflicting results with respect to the exact type of MMP's that are increased and no similar studies have been undertaken in rectal prolapse.

1.6 Biomechanical research in pelvic floor surgery

Having a framework that integrates how different factors interact to result in the development of rectal prolapse can facilitate understanding these complex causal questions relating to pelvic floor disorders. However finally prolapse results from a biomechanical failure of the tissue, resulting from failure of weakened tissues under normal forces, abnormal forces rupturing normal tissues or both pathways. Hence tissue biomechanical properties are fundamental to propagation of the disorder.

1.6.1 Biochemical composition and biomechanical properties

It is believed that most of the biomechanical properties determined from testing connective tissue are due to collagen. Mechanical properties of tissues are also dependent on the proportion of elastin these structures contain. Elastin allows the tissue to stretch and distend to a limited degree and to return to its original contour. Elastin, like collagen, is produced by the fibroblast. In some circumstances, elastin can elongate up to 70% of its length without rupture. In contrast, collagen can elongate only 4% prior to failure. Elastin tissue, however, forms only 5% by weight of

dermal connective tissue and is not thought to contribute significantly to its viscoelastic behavior.

It is commonly understood that many biochemical constituents are altered in prolapsed patients compared to non-prolapsed patients. For example, several studies have noted an increase in collagen and elastin content, collagen subtype III, and MMP activity of the rectum, vagina and its supportive structures (Keane et al. 1997; Warren et al. 1990; Chen et al. 2005; Karam et al. 2007). These alterations in biochemical properties have been examined in order to improve our basic understanding of how commonly cited risk factors (pregnancy, parity, menopause, and genetics) affect the rectum and its supportive tissues in the development of prolapse. While there is no doubt that such data are important, it is well known in other fields that biochemical changes can have a profound impact on the biomechanical properties of tissues (Jaasma et al. 2002; Liang et al. 2004).

It comes as no surprise, considering its structure and function, that pelvic floor tissue is viscoelastic/viscoplastic. This is important to consider when developing experimental protocols (Abramowitch et al. 2010). There are a variety of different viscoelastic tests that can be performed (Duenwald et al. 2009). Cyclic loading can be used to measure the area between loading and unloading curves, which describes the viscous phenomenon of energy dissipation, i.e. hysteresis. Other protocols including creep and stress relaxation are used to demonstrate how tissues can stretch out over time in response to an applied stress and how stress decays over time in response to an applied stretch, respectively (Cohen et al. 1976).

1.6.2 Biomechanical changes in pelvic floor disorders.

Biomechanics in the field of coloproctology is less intuitive when compared to urogynaecology where measurements of pressure, flow, volume, etc., have been used for decades in urodynamic assessments. Urogynaecology models allow for controlled studies, which are useful in understanding the underlining factors that may contribute to the development and progression of pelvic floor diseases in the colorectal context by systematically examining confounding risk factors. However, the need to translate findings to the clinic is very important, and therefore understanding how these models relate to humans must be evaluated.

A study compared the uniaxial tensile properties of vaginal tissue from prolapsed women and cadaveric specimens showing no signs of prolapse (Martins et al. 2013) . It was found that prolapsed and non-prolapsed vaginal tissue displayed a non-linear response typically found in biological soft tissues (Martins et al. 2013)This study also found large variations in the percent strain and stress at failure for each group; although not directly compared, there appeared to be little difference due to prolapse. A study examined how the biomechanical properties of women may change due to hormonal status by examining pre- and postmenopausal women (Vardy et al. 2005) ,A study by Goh showed that while the two groups did not differ in ultimate load or relative elongation (%), there was a significant increase in the elastic modulus of postmenopausal women (Goh 2003). A study by Lei et al. examined pre- and postmenopausal women with prolapse; this study also included pre- and postmenopausal controls (non-prolapsed) (Lei, Song, & Chen, 2007). In addition, this study limited the effects of potentially confounding variables by controlling for

age, BMI, and parity. Similar to Goh et al., this study found a trend between pre- and postmenopausal women (Feola et al. 2013). In addition, their data suggest that prolapsed women had a significant increase in the elastic modulus compared to non-prolapsed women regardless of hormonal status (Feola et al. 2013). This finding is interesting because most studies report that the vagina of prolapsed women has an increase in collagen type III (Keane et al. 1997; Mosier et al. 2010; Zhou et al. 2012), which has been found to decrease the tangent modulus of tissues. Clearly, these studies are important first steps in characterizing the biomechanical behavior of human vaginal tissue; however, no data is available to help us characterize the differences between normal and pathological tissues in rectal prolapse setting.

There is promising work using computational models that allow us to visualize the potential mechanisms of prolapse from a mechanical perspective, while providing quantitative data that cannot be obtained using the experimental testing methods discussed in this thesis. All of these areas of research are necessary as they provide a different, yet critical component, of a highly complex puzzle (Haridas et al. 2006).

Only the future knows what specific changes in treatment paradigms will result from biomechanics research in the field of pelvic floor surgery but, by building on the fundamental experiments that have already been established in these other fields, the translation of biomechanics research from bench-top to bedside in pelvic floor coloproctology can realized over an even shorter timeframe.

1.7 Hypothesis and Aims

Rectal prolapse occurs through a complex multistep process involving intrinsic structural factors. A tissue's ability to either generate or transfer forces is inherently coupled to its biochemical composition and spatial organization. Consequently, a deeper understanding of the biomechanics of the pelvic floor has the potential to shed new light on the mechanisms of pelvic floor disorders and our potential to treat them.

Rectal prolapse is likely to represent a failure of connective tissue support and, as the biomechanical properties of these connective tissues are determined by the composition of their connective tissues, failure could arise from changes with the tissues themselves, not only through imposition of abnormal forces these connective tissues. This sequence of events has been documented in the development of haemorroids (Willis et al. 2010), uterine prolapse (Paladini et al. 2007; Phillips et al. 2006) and stress urinary incontinence (Chen et al. 2002) . In these disorders collagen abnormalities have been the primary focus of research. In this thesis, we will focus on the passive mechanics of the skin and pelvic floor supportive tissues.

My hypothesis is thus that in patients that develop prolapse will either have an underlying connective tissue condition which will be apparent in tissues far from the site of injury (such as the skin (Holbrook & Byers 1989)), or that prolapse arises from initial injury of the pelvic floor with consequent attempts at repair leading to progression of the prolapse.

This hypothesis will be tested by taking pelvic connective tissue and skin samples from patients with prolapse and from control patients with no clinical evidence of the disease. The specific areas for study will include:

1. Analysis of tissue histologically to assess whether there are differences in the extracellular matrix of the skin and prolapse tissue in patients with prolapse compared with patients with no prolapse (controls), concentrating on elastic fibre expression and organization and expression of MMPs.

2. Mechanical testing of skin and prolapse tissue to determine differences in tissue properties between prolapse patients and control patients.

3. Culture of cells from skin of prolapse patients and control patients to examine expression of extracellular matrix genes and of proteases.

Results will be related to grouping of patients in relation to sex, age, prolapse type (internal or external) and hypermobility status. The results should show whether there is any inherent extracellular matrix change in prolapse patients or a subgroup of prolapse patients relative to controls, and whether prolapse type is related to factors involved in matrix turnover and degradation.

CHAPTER 2

METHODS

2.0 Methods and Materials

2.1 Patient And Animal Tissues

2.1.1 Patient Characteristics

2.1.1.1Prolapse group

Patients with rectal prolapse were recruited on a prospective basis from elective theatre lists of the Oxford Pelvic floor centre. They underwent informed consent and were allocated a patient study number. (See Appendix 2 for patient record and consent pages). All patients were treated with a laparaoscopic ventral rectopexy. Following the procedure all patients were followed up for 12 months and all additional procedures related to their disease were noted.

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 daefacation or chronic perineal/abdominal pain. The young female group were all nulliparous women. The standard diagnostic work up is outlined below in Table 2.1.

Table 2.1.Presurgical diagnostic tests.

Test	**Purpose**
Colonoscopy/Flexible Sigmoidoscopy	To exclude malignancy or other pathology
Defecating proctogram	To identify internal prolapse and enterocele
Colonic transit study	To identify patients with genuine slow transit who will need alternative treatment.
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 ODS and non-diagnostic proctograms.
Pelvic Floor Questionnaire's including Faecal Incontinence Severity Index, Fecal Incontinence Quality of Life scale, and Gastrointestinal Quality of Life Index	To evaluate symptoms objectively at baseline

2.1.1.2 Control Group

Patients undergoing emergency surgery for appendicitis, cholecystitis or elective surgery for colon cancer were recruited into the control arm of the study. They underwent informed consent and were also allocated a patient study number. Details are given in Table 2.2.

2.1.1.3 Animal Tissues

Bovine tissue from 18-month-old steers was obtained from Witney abattoir. The distal foot was procured, placed in bleach to sterilize it, then dissected and dermis was harvested. Dermis was digested overnight and fibroblasts were the cultured for 48-72 hrs. Skin biopsies were taken and used for immunohistochemical and mechanical analysis. Porcine tissue was taken from 6-month-old piglets at Witney abattoir. Skin biopsies were taken and processed for further testing.

2.1.2 Tissue sampling from Patients and Controls

2.1.2.1 Skin Sampling from Patients.

Skin samples were obtained during laparaoscopic surgery just prior to laparoscopic port site insertion. Three skin specimens were taken during each procedure; 1 x 10 mm from the umbilical port and 2 x 5mm biopsies other port sites. See figure 2.1.



Figure 2.1. Laparoscopy port placement with arrows indicating site of sample acquisition

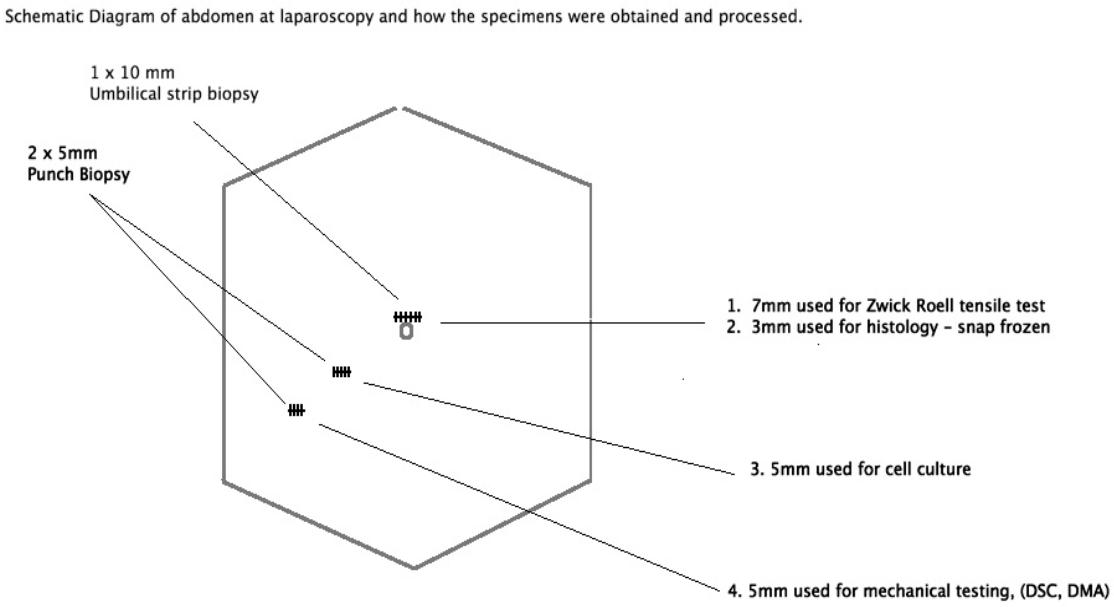


Figure 2.2: Schematic diagram of abdomen looking down at the patient on the table at laparoscopy showing which specimens were obtained for testing.

2.1.2.2 *Sampling Pelvic Tissues*

The pelvic tissue sample acquisition was completed during a key stage of the laparoscopic ventral mesh rectopexy procedure. Using a diathermy device at the level of the sacral promontory, it was then continued down the right pelvic sidewall. 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 sidewall and the front of the rectum. See figures 2.3 and 2.4

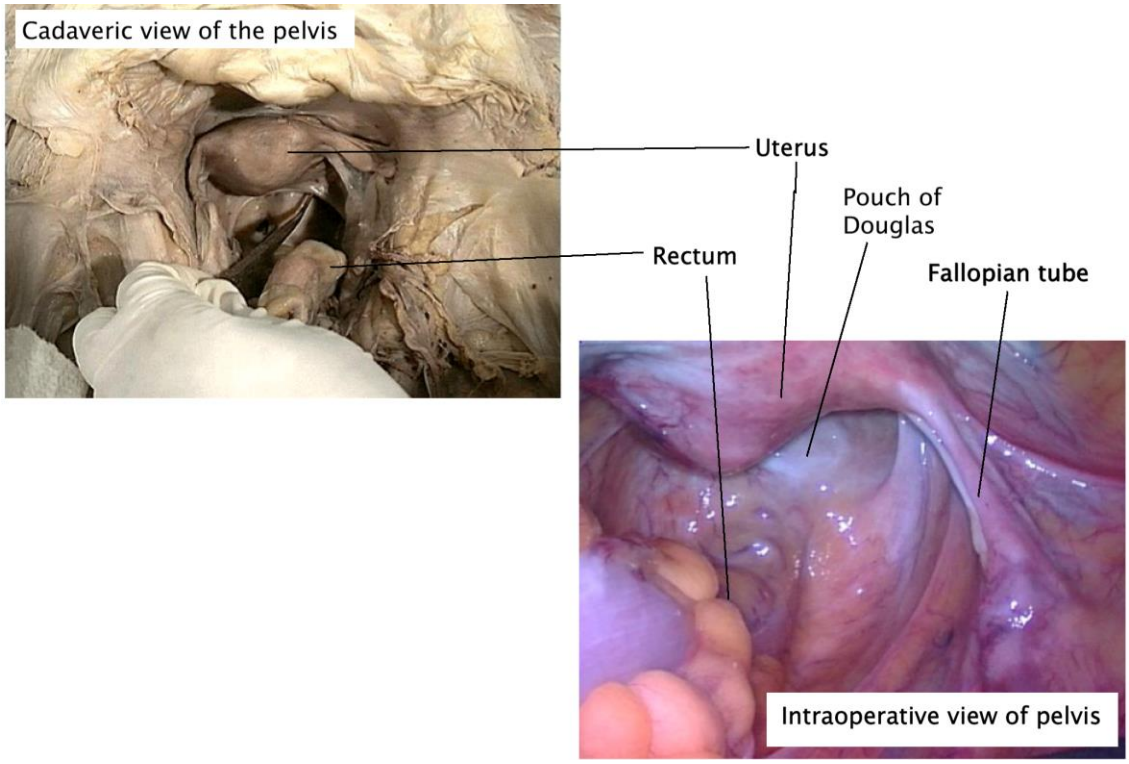


Figure 2.3: Cadaveric specimen of intraoperative view at laparoscopy. Pouch of Douglas excised as pelvic specimen

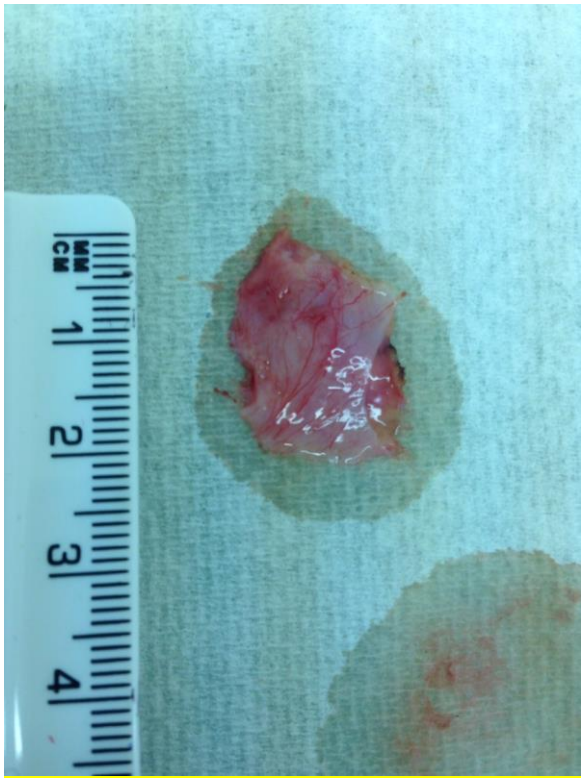


Figure 2.4: Typical example of tissue removed from pouch of Douglas during laparoscopic ventral mesh rectopexy

2.2 Tissue Processing

Human Tissue was collected within 2-6hrs of surgery and divided as shown in Table 2.2

Table 2.2 - Division of tissue samples

No of specimens	**Sample used for**	**Treatment method**
1	Snap frozen in hexane solution and stored in -80 ^{o}C.	(i)
1	Fixed in formalin and stored for histological processing	(ii)
1	Differential scanning calorimetry	(iii)
1	Dynamic mechanical analysis	(iii)
1	Zwick tensile testing	(iii)
1	Cell culture	(iv)

(i) One sample was immediately snap frozen and transferred to the Department of Anatomy, University of Oxford in dry ice and placed in sealed cryotubes at -80 ^{o}C. (ii) Tissue in formalin was allowed to fix for a minimum of 48 hours. (iii) Samples used for mechanical testing were kept moist in a humidified sealed tube and processed immediately to negate any adverse effects of storage. (iv) Sample for cell culture was placed in DMEM and cultured as per 2.9.1



Figure 2.5: Typical examples of samples of tissue from umbilical 10mm port site taken at laparoscopy from one patient with a ruler indicating size (arrow denotes 1cm).

2.3 Histological Staining for visualization and localization of tissue components

The samples in formalin were taken to the histology processing facility at the Dunn School of Pathology, University of Oxford. Processing was performed by the technician in the department (RS). Once fixed the samples were removed and prepared for processing and embedded into wax blocks, through substitution of water in the specimen for wax. Since water and wax are immiscible the process involves graded exchanges of water for alcohol and then the substitution of alcohol for xylene. Xylene is then substituted for wax and then the specimen is embedded (Clayden EC 1976). The embedding phase is a critical step as the specimens must be orientated in

a known and desired plane. Once correctly orientated the specimens were set in molten wax. Sections were cut onto vectabond-coated slides. The use of coated slides was important in preventing specimen loss during the many antigen retrieval steps.

2.3.1 *Histological Staining Methods*

2.3.1.1 *Hameatoxylin and Eosin (H&E)*

All samples were stained using the H&E, the technique used frequently for general observations of organization of tissue section (Kiernan et al. 2010). In this project Harris Haemotoxylin was used and neutral eosin as a counterstain. (Leica Microsystems (UK) LTD)

2.3.1.2 *Elastin Orcein staining*

Elastin staining was performed using the traditional technique of hydration, orcein stain and counterstain with Harris haemotoxylin and acid alcohol followed by dehydration (Kiernan et al. 2010)

2.4 Immunohistochemistry

Immunohistochemistry utilizes 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; appropriate antibodies are produced and subsequently collected. In order to then visualize 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 binding sites for the staining molecules to bind to. The eventual staining solution maybe tagged with an immunofluorescent probe or conventional dye that can be viewed using standard microscopic techniques. In this study, tissue was cryopreserved or fixed in neutral buffered formalin solutions. Where tissues were fixed with formalin, an antigen retrieval process was utilised with a commercially available kit (G. Liu et al. 2006). The avidin-biotin complex (ABC method) was employed for staining in most cases. (Thermo Fisher Scientific , MA USA)

2.4.1 *Immunohistochemistry staining*

Table 2.3. – Antibodies and reagents used for immunohistochemistry staining

Antibody	Dilution	Supplier	Catalogue No,
Monoclonal mouse Anti-Fibrillin1	1:50	Abcam, Cambridge, MA, USA	ab124334
Monoclonal mouse Anti-Fibulin-5	1:100	Abcam, Cambridge, MA, USA	ab198212
Normal donkey serum	1:10	Sigma, Saint Louis, USA	d9663

2.4.1.1 Protocol used for frozen sections

The methods used were adapted from that described in the literature

Briefly, skin sections (20μm) were cut on a cryomicrotome from snap-frozen samples, washed with Tris buffer (50 mM Tris-HCl + 10 mM calcium acetate, pH 7.2) 3 times for 3 minutes each time. Sections were then blocked with 10% normal donkey serum in Tris buffer (McEuen AR et al., 1995) for 45 min at room temperature.

The first primary antibody was then applied directly without further washing of the sections after the blocking solution was removed, and incubated at 4 °C overnight. After washing 3x 3min with PBS, sections were incubated with the first secondary antibody (raised in donkey) (Paladini et al. 2007) conjugated with Cy3 (AbCam MA USA Cat no: ab97035) at room temperature for 45 minutes.

After washing again with PBS (3x3minutes), sections were blocked again with 10% normal donkey serum, then incubated with the second primary antibody at 4 °C overnight. The sections were then washed 3x3min with PBS, and incubated with the second secondary antibody (Paladini et al. 2007) conjugated to daylight-488, at room temperature for 45 minutes. Sections were then washed and mounted with mounting medium (Vector Laboratory, Peterborough, UK Cat No: H1500).

2.4.1.2 Protocol used for fixed section from paraffin blocks

The method used was adapted from Sato et al (Sato et al. 1991). Briefly, four-micrometer paraffin sections were melted overnight at 37°C, cleaned in xylene, and hydrated in decreasing grades of ethanol. After blockage of endogenous peroxidase activity with a solution of hydrogen peroxide and methanol, an antigen retrieval step was performed using citrate buffer and a steamer for 30 minutes (Markova et al. 2003)

For a negative control, normal mouse serum (Sigma-Aldrich Ltd, Dorset UK) was substituted for the antibody. Slides were sequentially treated with the primary mouse

antibody, biotinylated anti-mouse immuno- globulin AbCam MA USA Cat no: B7264 , and Streptavidin–biotin-peroxidase complex (LSABTM+/HRP kit, Dako, Carpinteria, CA). Diaminobenzidine (Vector Laboratory, Peterborough, UK Cat No: SK-4100) was used as chromogen in the presence of hydrogen peroxide. Slides were then counterstained with haemotoxylin. All reactions were carried out at room temperature (22°C).

2.5 Specimen Analysis; *Assessment using a scoring system.*

Experiments were conducted sequentially. Two independent reviewers who were blinded to the patient information viewed slides on light microscope. (FS, AS). Slides were graded according to the intensity of the staining. The grading system used was as shown in Table 2.4.

Table 2.4: Grading system used for immunohistochemistry analysis

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 <50% of total section area.
Grade 3	Strongly positive staining which occupies a total of >50% of the total section area.

2.6 Specimen Analysis; *Assessment using Image J*

Slides were viewed using a light microscope. Digitised images were captured using a microscope-mounted camera. These images were saved as high-resolution tiff files. This image was then uploaded onto an Image analysis software programme (Image J, obtained from: Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). The image threshold was adjusted such that only the darkly stained elastic fibres were visualised. The area occupied by these fibres was then calculated in a manner previously utilised by others (Uitto et al. 1983)

2.7 Biomechanical Testing

2.7.1 *Differential Scanning Calorimetry*

Differential Scanning Calorimetry (DSC TA Instruments Q2000 ™)

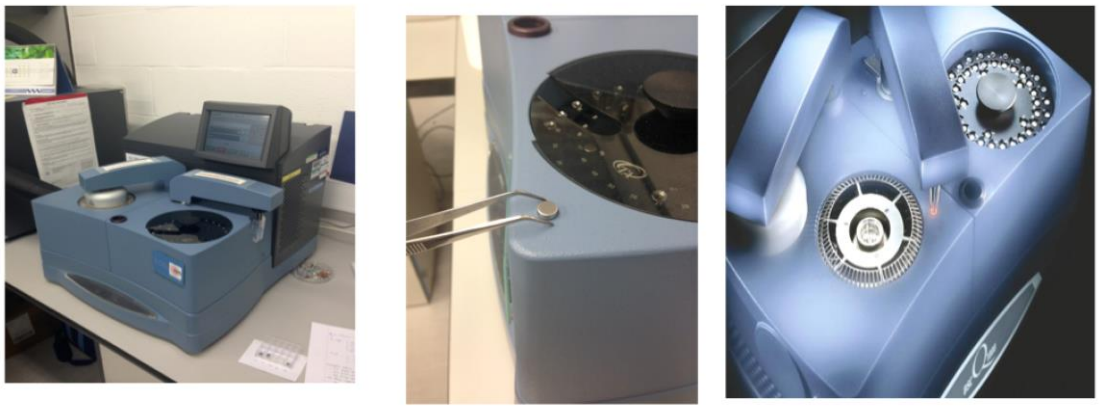


Figure 2.6: Differential scanning calorimetry machine showing details of the loading pan and arm.

Differential scanning calorimetry or DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature.

Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. The reference sample is chosen to have a well-defined heat capacity over

the range of temperatures to be scanned. Here, the heat capacity calibration was performed with Sapphire standards, and Indium was used for temperature calibration.

Specimens were placed in hermetically sealed conventional Tzero™ DSC pans (DSC Consumables, Austin, MN, USA) to prevent any liquid loss during heating and hence to avoid changes in thermal behavior. Samples were subjected to a modulated temperature ramp between 20 and 80°C at a rate of 2°C /min with 0.318°C amplitude of modulation and results recorded in a typical thermogram (Figure 2.7).

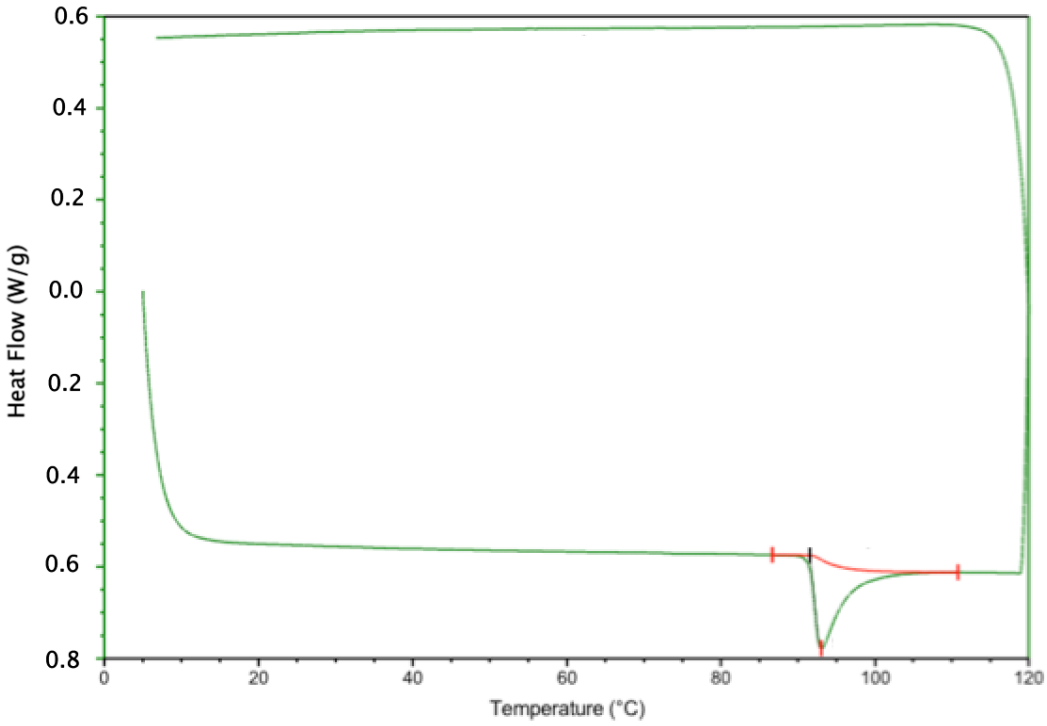


Figure 2.7: a typical thermogram

All DSC-related thermal parameters were obtained from thermograms as shown in Chapter 3 (Figure 3.1). The T_{onset}, T_{peak} and the denaturation enthalpy (DH) were measured, and are all related to degrees of denaturation (Poland 2001). T_{onset} (°C) is defined as the onset of the endotherm on the thermogram. This is proposed to

represent breaking of the water-amide hydrogen bonds, resulting in increased mobility of the protein chain backbone and resulting in an ordered-to-disordered transition (Poland 2001).

The T_{peak} (°C) is the peak of the endotherm, and reportedly corresponds to the temperature at which the majority of proteins fall out of solution. The denaturation enthalpy, DH (J/g), was calculated as the area under the heat absorption curve by using two-point peak integration. DH is a measure of the energy required to fully denature all the collagen present and is thought to correspond to the relative amount and distribution of triple helical structure within the sample (Liu et al. 2000).

2.6.2 Dynamic Mechanical Analysis

Dynamic mechanical analysis or DMA is a technique used to study and characterize materials and was applied to skin samples using a Dynamic Mechanical Analysis DMA Q800 (TA Instruments, New Castle, USA.) as shown in Figure 2.8 Dynamic Mechanical Analysis DMA Q800

A sinusoidal stress is applied to the samples and the strain in the material is measured, allowing one to determine the complex modulus. The frequency of the stress can be altered, leading to variations in the complex modulus; this approach can be used to identify transitions to the rotation of the entire specimen or the rotation of parts of the specimen relative to one another in torsional motion (Gennisson et al. 2010).

Specimens were loaded onto the DMA tester and subjected to continuous and intermittent forces. The modulus was then calculated from the curves by device specific software.

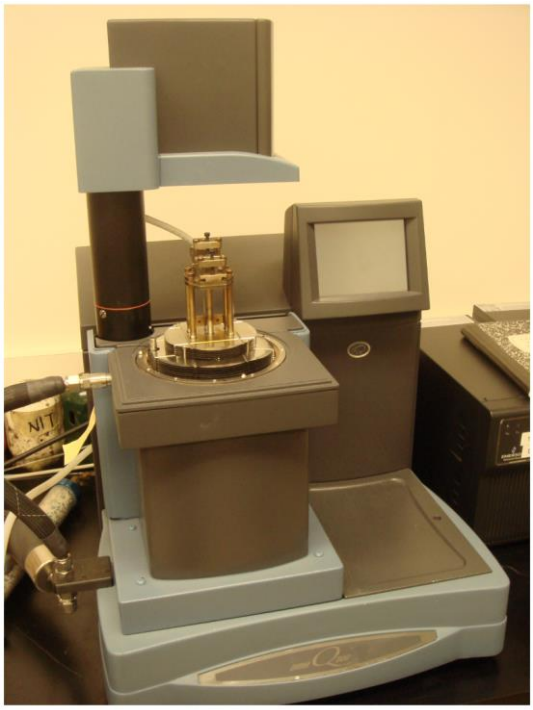


Figure 2.8: Photograph of Dynamic Mechanical Analysis tester DMA Q800.

2.6.3 *Zwick Roell* ™

A Zwick Roell industrial machine (Zwick Ltd, Ulm Germany) (Figure 2.9) was used with custom-built ceramic clamps, specifically designed to hold clinical tissues while minimizing slippage. Width and depth of samples was measured at three points on the specimen using electronic calipers and the mean width and depth was calculated and recorded. Samples were clamped in the custom grips and a force of 1N/min was applied until a S_{max} (maximum force before breaking point) was reached (Figure 2.9a). The area under the curve was used to calculate the Young's modulus.

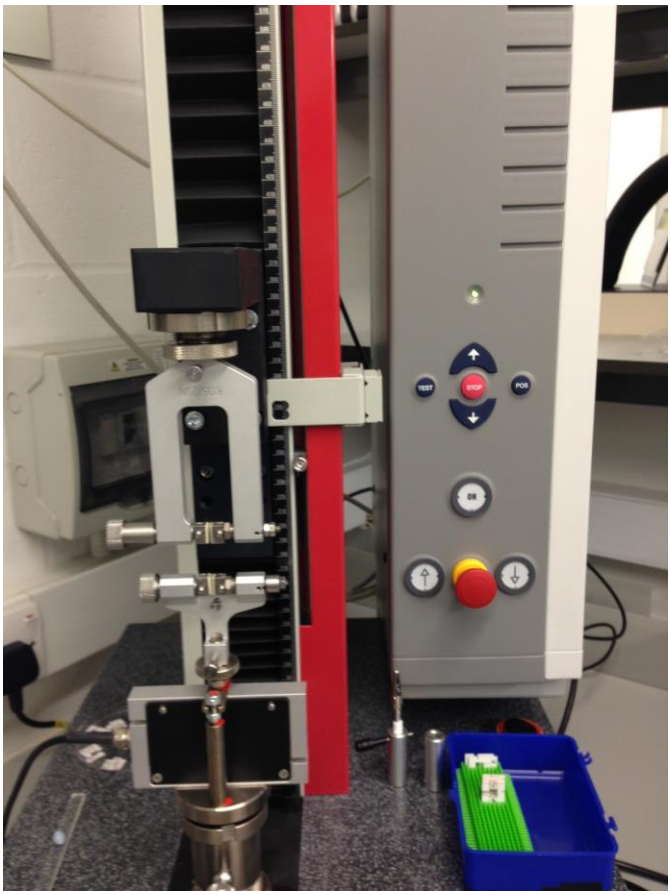


Figure 2.9. Photograph of the Zwick Roell machine used for testing skin specimens.

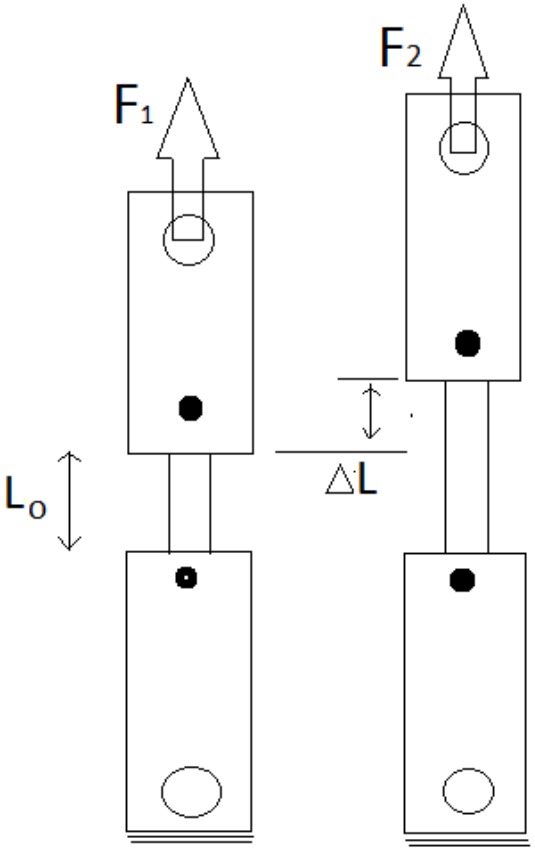


Figure 2.9a: Schematic diagram demonstrating the tensile test methodology of the Zwick Roell machine. Samples were clamped in the custom grips and a force of 1N/min was applied until a $S_{max,}$ the breaking point, was reached.

2.8 Polymerase Chain Reaction (PCR)

2.8.1 *RNA Extraction*

Total RNA extraction from cultured cells was accomplished using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction, which included an on-column DNase treatment step. RNA quality was measured using a Nanodrop spectrophotometer (Agilent Technologies, Santa Carla, USA) and reverse transcription was performed using the Nanoscript2 RT kit from Primerdesign Ltd, (York UK) as per manufacturer's instruction. Negative controls (RT) were carried out in the absence of reverse transcriptase and used to check for genomic contamination. All samples were stored at -20°C prior to PCR amplification (Ma et al. 2013).

Total RNA was isolated from cells using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, after cell lysis with RLT-Buffer containing 1% mercaptoethanol equal volume of 70% ethanol was added and transferred to the RNeasy columns. Columns were washed once with RW1-Buffer and DNA was digested directly at the column using RNase free DNase I (Qiagen, Hilden, Germany) for 15 minutes to avoid genomic DNA contamination. Subsequently, columns were washed again with RW1-Buffer followed by two washing steps with RPE-Buffer. Finally, the total RNA was eluted using water (Ma et al. 2013).

The yield of purified total RNA was determined by measuring the UV absorbance at 260 nm on the Nanodrop ND1000 spectrophotometer. cDNA was obtained from 250 ng total RNA using the high-capacity kit (Applied Biosystems, Foster City, USA) according to the manufacturer's protocol and finally diluted to a concentration of 1.25 ng/ μ L for further analysis. To assess the mRNA expression of the target genes real-time PCR was performed using 5 μ L of the gene expression master mix (Applied Biosystems) and the 0.5 μ L of the gene expression assay for Fibulin 5 (Hs00164004_m1), MMP1 (Hs00233958_m1), MMP9 (Hs00234579_m1) and TIMP-1 (Hs00122992_m1) (each includes forward and reverse primers as well the fluorescently FAM-labeled probe) from Applied Biosystems and 1 μ L of cDNA in a final volume of 10 μ L (Ma et al. 2013).

2.8.2 *GeNorm*

GeNorm is a popular algorithm used to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel. From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes (Wang et al. 2010). We used this algorithm to define housekeeping genes for our study.

2.8.3 *Primers.*

The housekeeping genes β -actin and GAPDH served as references to which the gene of interest was normalized [quantitative PCR (par) studies]. Primers for all genes were designed to allow amplification of exon-spanning amplicons, thereby allowing for

identification of contaminating genomic DNA. Primers for all genes tested (Chapter 6, Table 3.3) were synthesised by PrimerDesign Ltd. (York, UK)

2.8.4 *Polymerase Chain reaction assessment of expression of genes of interest.*

Non- or semi-qPCR was performed using DNA polymerase (Primer Design Ltd) or). The two polymerases have slightly different amplification profiles: an initial denaturation step at 94°C followed by 35 cycles of 94°C for 30 s and an annealing temperature specific for each primer set for 30 s and 72°C for 1 min (Primer Design Ltd) or an initial denaturation step at 95°C followed by 35 cycles of 95°C for 30 s and an annealing temperature specific for each primer set for 30 s and 72°C for 30 s; the reaction was completed with a 5-min extension step. β -Actin or GAPDH served as internal control.

2.8.5 Quantitative polymerase chain reaction of gene expression

Quantitative analysis of mRNA expression was determined using Brilliant SYBR Green qPCR Master Mix (Primer Design Ltd). Duplicate reactions were performed in 25-μl volumes including 1.5 μL of cDNA, 12.5 μL of SYBR Green qPCR Master Mix, and 0.75 μL of the passive reference 5-carboxy- X-rhodamine (ROX) dye (supplied with Master Mix). The following cycling conditions were used: initial denaturation at 95° C for 10 min (*segment 1*) followed by 50 cycles of 95°C for 30 s,

annealing at 60°C for 1 min, and extension at 72°C for 30 s (*segment 2*). A final segment (*segment 3*) of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s completed the protocol.

Fluorescence data were collected and used for analysis from data acquired at *segment 2*. Data (means ± SE) are expressed as $2^{\Delta C_t}$. All quantitative data were accrued from three different samples. Melt curve analysis was performed to ensure that each primer set amplified a single product; because SYBR Green will bind any double-stranded product, any amplification of nonspecific material will artificially increase the level of fluorescence. A single peak confirmed that a single product had been amplified and that the C_t (cycle threshold) values generated were accurate. C_t values were determined using Biogazelle qPCR software (Primer Design Ltd), and RNA abundance relative to β -actin or GAPDH was calculated using $2^{\Delta\Delta C_t}$.

Standard curves were plotted using 10-fold serial dilutions of cDNA from murine brain (deemed a suitable positive control from previous semi-qPCR) to ascertain the efficiency of amplification of each gene used in this study. After a logarithmic conversion, a linear response was generated, and only R^2 values ≥ 0.99 were accepted. Additionally, it was ensured that efficiencies were comparable between each primer set in each individual experiment.

2.8.6 Reagents and Suppliers

Reagents and chemicals in this thesis were purchased from Invitrogen (Paisley, UK) unless otherwise stated. Tissue culture flasks, plates and tubes were obtained from Corning (New York, USA). Cell culture media and antibiotics were produced by, Invitrogen.

2.9 Cell Culture

2.9.1 *Dermal Fibroblasts*

Dermal Fibroblast cells were stored cryogenically at Department of physiology, genetics and anatomy (DPAG), University of Oxford

Human skin specimen were placed in 6 well plates and fibroblasts were cultured in complete Dulbecco's modified eagles medium (DMEM no 30 2002) supplemented with 10% fetal bovine serum (FBS)) Biosource, Nivelle, Belgium), 0.5% Ascorbic Acid and 1% penicillin/streptomycin (Invitrogen) in 75cm^2 vented tissue culture flasks with 10ml of medium. All cells grew adherent to the flask. Cells were incubated at 37°C in a humidified 5% CO_2 environment (Heracell 150 incubator, Thermo Scientific, Massachusetts, USA) and were grown to 70 – 80% confluence before the next passage or experiments.

2.9.2 *Sub-culture of dermal fibroblasts*

Cells were subcultured when they reached 70-80% confluence. Complete medium and 0.5% trypsin, diluted in phosphate buffered saline (PBS) (Invitrogen), was prewarmed to 37°C. The growth medium was removed and the cells were first washed with 1 x PBS. This was followed by trypsinisation using 3 ml of 0.5% trypsin and the cells were incubated at 37°C for 1 – 5 minutes. Gentle tapping encouraged the cells to become dislodged. 10mL of complete medium were added to neutralize the trypsin and the cell suspension was gently pipetted up and down against the flask wall multiple times to break cellular clumps. The cells were then centrifuged at 1500 rpm for 5 minutes, the supernatant removed, and the cell pellet resuspended in 10ml of complete medium. The cells were then split into 1:10 or 1:20 dilution. Cells required subculturing once every 5 to 7 days.

2.9.3 *Cryo-preservation and resuscitation of cells from liquid nitrogen*

To prepare cells for liquid nitrogen storage, 1 x10^6 cells were centrifuged at 1500rpm for 5 minutes and the supernatant removed. The cell pellets were resuspended in 1 ml FBS containing 10% dimethyl sulfoxide (DMSO) and aliqouted into sterile cryogenic vials (Corning, USA), placed on dry ice for 90 minutes before storage in a CBX40 cryogenic freezer (Wessington Cryogenics, Houghton le Spring, UK) within the vapour phase of liquid nitrogen at -150°C. To thaw cells, the vials were placed under running warm water until defrosted, then immediately transferred into 10ml of DMEM with 10% FBS and placed in a 75cm^2 flask for incubation at 37°C.

2.9.4 *Cell counting*

A manual count was performed using a haemocytometer (Weber Scientific) and a BH2 light microscope (Olympus, Southend- on – Sea, UK). A 20µl cellular suspension was mixed into 20µl of 0.4% Trypan blue in an Eppendorf tube. A coverslip was attached to the haemocytometer and 20µl of cell/Trypan blue mixture was pipetted beneath the cover slip so that it filled the chamber by capillary action avoiding any air bubbles to be present. The haemocytometer was then placed on the microscope stage and using a x10 objective lens, all cells (stained and unstained) located within 20x20 small squares (= 5 x 5 medium squares) were counted using a tally counter.

Viable cells remain unstained and non-viable cells stain blue. The following calculations were then performed:

Total number of cells/ml = [Total number of cells x2 x 10^4]

% viability = [unstained cells/total number of cells] x 100

2.9.5 *Preparation of cultured cells for immunohistochemistry*

For immunohistochemistry of cultured cells, single cell suspensions of dermal fibroblasts were obtained as above. Cells were pelleted by centrifugation and washed twice with PBS and resuspended to concentration of 5×10^3 in each 96 well plate. An oval area was marked out using a hydrophobic ImmEdge pen (Vector Labs) on polylysine coated microscope slides and left to air dry for 10 minutes. A drop of cell suspension, containing around 5×10^3 cells was plated on the oval area and left to attach to the microscope slide at room temperature for 30 minutes. Cells were then fixed with -20°C methanol for 5 minutes, then 2% formaldehyde in PBS for another 5 minutes. The slides were then washed twice with PBS for 5 minutes each, and once with PBS-Tween (PBS-T) for 5 minutes. Cells were then blocked using Invitrogen blocking agent (0.1g/10ml PBS-Tween) for 30 minutes, before incubating with the primary antibody.

2.9.6 Scoring Systems

2.9.6.1 Assessment of connective tissue status

All patients recruited to the study were clinically assessed using the beighton scoring system to determine whether they fulfilled the criteria for benign joint hypermobility syndrome and to assess the laxity of their joints (Smits-Engelsman et al. 2011).

Table 2.5: Beighton scoring system

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

A beighton score of >4/9 and symptoms of arthralgia in >4 joints for three months was indicatory of benign joint hypermobility. A beighton score of 1-3 and arthralgia in 1-3 joints or history of joint dislocation or hernia were given a grading of minor features of joint hypermobility.

Prolapse severity was assessed with Oxford prolapse grading system (Wijffels et al. 2010).

Table 2.6 Oxford prolapse grading system.

Grade	Anatomical Description
1	Full-thickness infolding of rectal wall
2	Prolapse of rectal wall to the top of the rectocele
3	Prolapse of the rectal wall to the top of the anal canal
4	Prolapse of the rectal wall into anal canal
5	Prolapse of the rectal wall beyond anal verge (external prolapse)

RESULTS

CHAPTER 3 ANIMAL STUDIES

3.0. Animal studies.

3.1.1 Introduction

As pelvic floor surgery begins to become more relevant in an aging population, there is the potential to recognize the benefits of rigorous biomechanics research to improve the patient care.

While there is clearly much to learn regarding the biomechanics of rectal prolapse, a lack of significant progress thus far can at least be partially attributed to the difficultly of performing controlled, longitudinal studies in human patients along with the ethical issues surrounding the procurement of human tissue. This can be especially problematic in biomechanics research since larger tissue samples are frequently required to perform experiments appropriately. Animal models, on the other hand, afford the opportunity to test hypotheses more rigorously in a controlled environment. The Pig model was chosen in this set of experiments (see chapter 2.1.6)

3.1.2 Porcine Feasibility studies in mechanical testing of skin samples

The analysis of an up-to-date knowledge suggests that mature porcine skin shows the greatest similarity to human skin both in terms of structure and mechanical properties (Godin & Touitou 2007; Liu & Yeung 2006). There is a particularly close similarity between the thickness of the individual layers of the skin of pig foetuses used in the studies and the corresponding integuments in humans (Barbero & Frasch 2009;

Cilurzo et al. 2007; Harunari et al. 2006; Shergold et al. 2006; O'Grady et al. 2000; Sullivan et al. 2001).

Biomechanics in the field of coloproctology is less intuitive when compared to urogynaecology where measurements of pressure, flow, volume, etc., have been used for decades in urodynamic assessments.

Tissues can be generally divided into two subgroups: (1) active tissues, i.e. those capable of generating a force (e.g. striated, smooth, and cardiac muscle), and (2) passive tissues, i.e. those capable of transferring forces (e.g. the ECM of connective tissues). For both subgroups, the tissue's ability to either generate or transfer forces is inherently coupled to its biochemical composition and spatial organization. Consequently, a deeper understanding of the biomechanics of the pelvic floor has the potential to shed new light on the mechanisms of pelvic floor disorders and our potential to treat them (Goh 2003).

3.1.3 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was performed as described in chapter 2.7.1

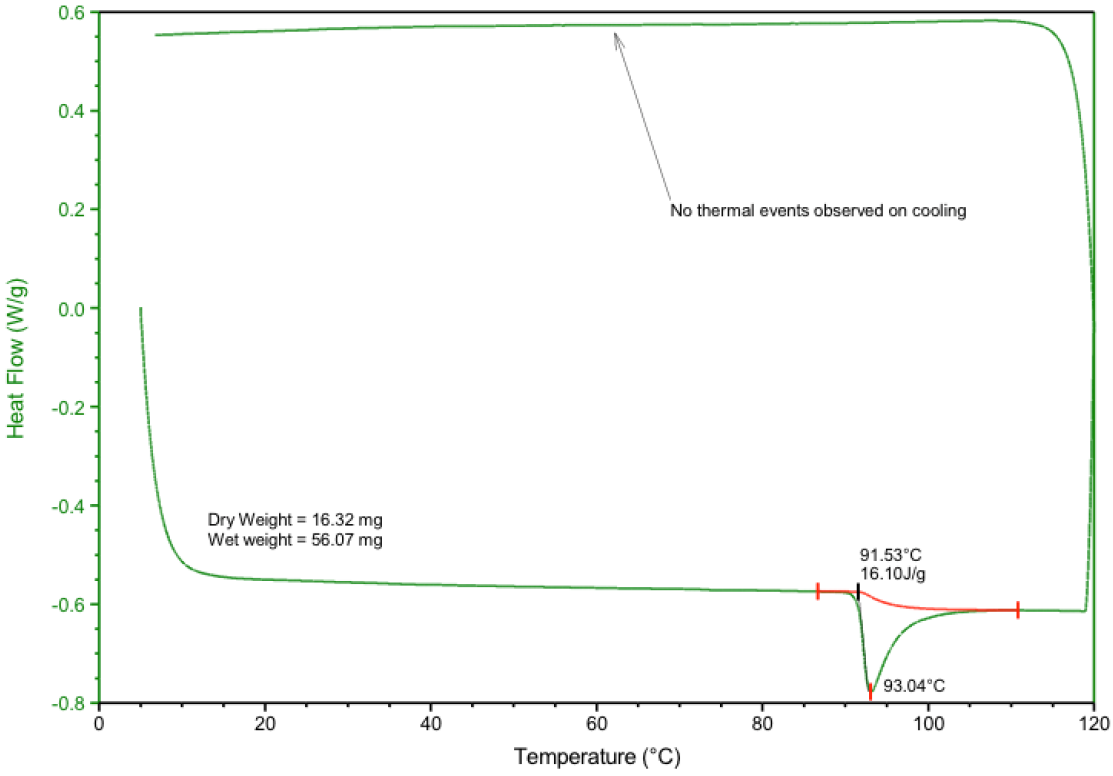


Figure 3.1. A standard differential scanning calorimetry curve. As the temperature increases in the x –axis there is a quantifiable heat flow change. In this example the sample is denatured at 91.53 C with a maximum denaturation at 93.04 C. The total area under this curve is calculated as the total energy required for denaturation, in this example worked out as 16.10J/g. The sample is then cooled at a rate of 2C/min. Any further energy changes are not observed.

3.1.3.1 Differential Scanning Calorimetry of Porcine Skin

Three repeat biopsies were taken from 10 different pigs to assess the reproducibility of differential scanning calorimetry on healthy samples of a similar age; it was not possible to obtain human samples to obtain such data. The mean sample enthalpy (which is calculated as the area under the curve) of repeat samples from the same pig

ranged from 5.82+/-1.16 to 6.96 +/- 1.33. The mean sample enthalpy of samples from different pigs was 6.11 – 6.19 +/- 0.31 -0.42. The overall mean enthalpy was 6.15 +/- 0.36.

Porcine Tissue	Enthalpy Sample 1	Enthalpy Sample 2	Enthalpy Sample 3	**Mean**	Std Dev
1	5.81	5.72	5.94	**5.82**	1.16
2	6.12	6.03	6.25	**6.13**	1.25
3	5.93	5.72	6.01	**5.88**	1.29
4	7.20	6.93	6.75	**6.96**	1.33
5	5.75	5.84	5.90	**5.83**	0.37
6	5.90	5.83	5.69	**5.81**	0.11
7	6.01	6.20	6.61	**6.27**	0.31
8	5.87	5.90	6.10	**5.96**	0.92
9	6.39	6.41	6.20	**6.33**	1.19
10	6.51	6.60	6.40	**6.50**	1.56
Mean	**6.14**	**6.11**	**6.19**		
Std Dev	0.42	0.38	0.31		

Table 3.1. Enthalpy numbers obtained from porcine feasibility study.

The feasibility study highlights the low standard deviation recorded in between samples from different pigs and thus any variability seen in the human studies is likely to be significant.

3.1.3.2 Dynamic mechanical analysis of Porcine Skin

Two repeat biopsies were taken from 5 different pigs to assess the feasibility and comparability of dynamic mechanical analysis (DMA) as a mechanical test. The mean Young's modulus of repeat skin samples from the same pig was 2.50 – 3.39 +/- 0.15 - 0.33. The mean Young's modulus of skin samples from different pigs was 2.80- 3.10 +/- 0.38- 0.48. The overall mean was 2.91 +/- 0.41

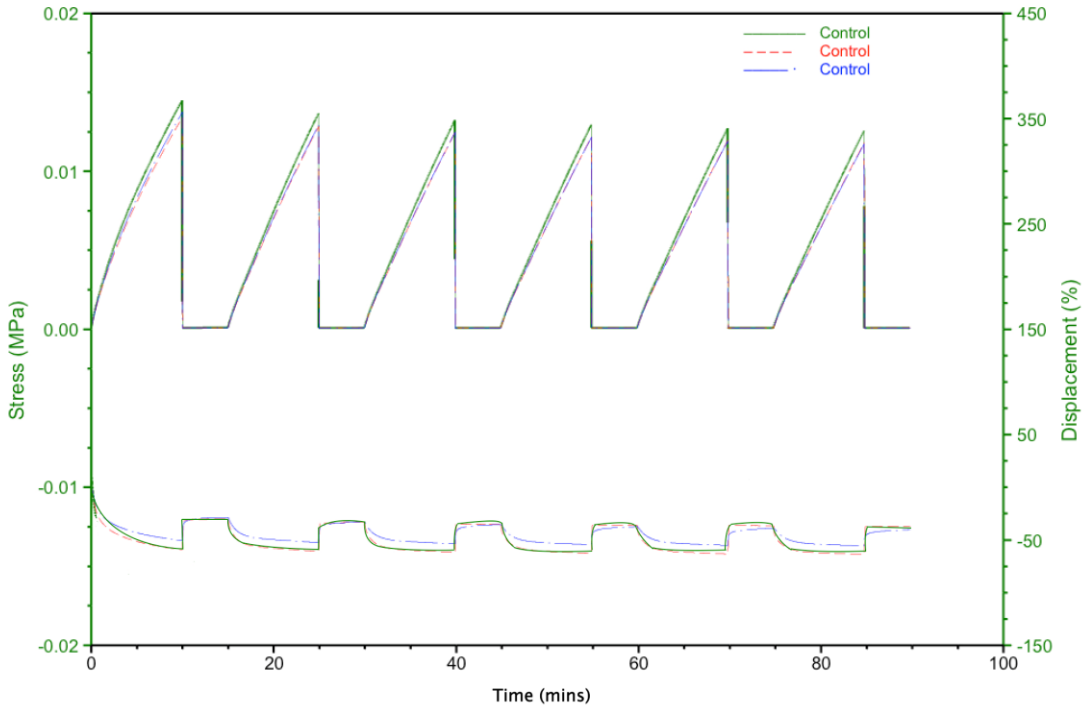


Figure 3.2: A typical dynamic mechanical analysis curve. A stress is repeatedly applied and the strain is measured simultaneously with measurement of stress

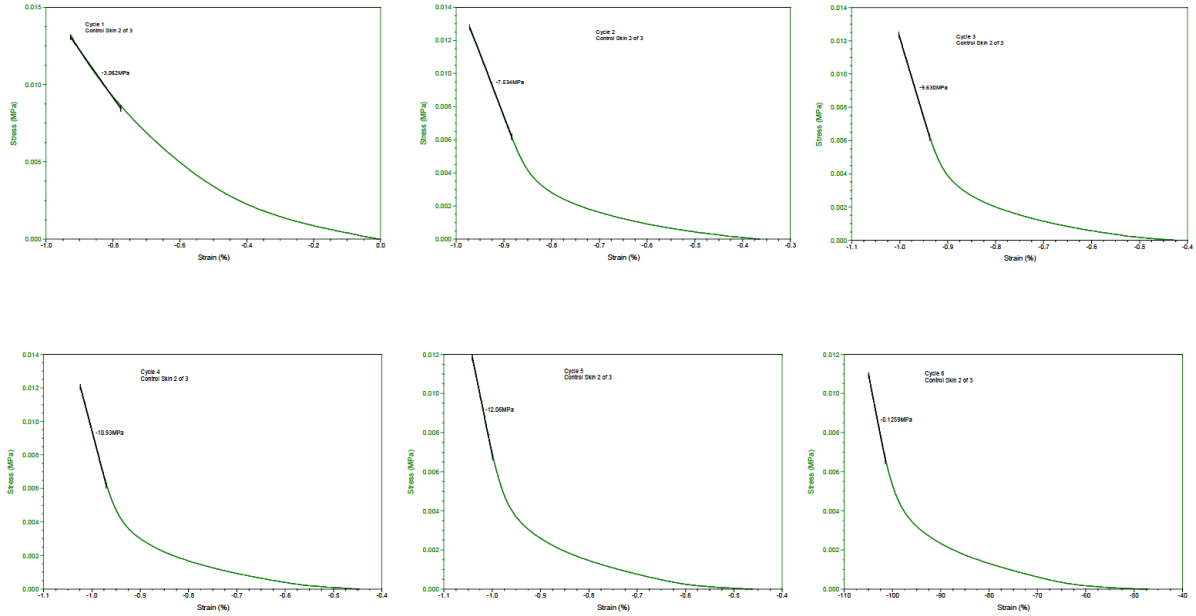


Figure 3.3: A typical stress strain curve obtained during one cycle of the dynamic mechanical analysis, and which is subsequently used to calculate the Young's modulus.

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Porcine Tissue	Young's Modulus MPa Sample 1	Young's Modulus MPa Sample 2	**Mean**	% Difference
1	2.11	2.89	**2.50**	2.67
2	3.06	2.90	**2.98**	2.64
3	3.20	3.58	**3.39**	3.34
4	2.77	3.05	**2.91**	2.81
5	2.90	3.11	**3.00**	2.86
Mean	**2.80**	**3.10**		
Std Dev	0.42	0.38		

Table 3.2 Young's modulus calculated from results of Dynamic mechanical analysis of porcine skin.

3.1.3.3 Zwick Roell tensile test

The biomechanical properties of tissues are important because they provide quantitative information regarding the mechanism by which tissues function to either generate or respond to physiological forces. Additionally, these measurements can provide insight into pathology and the efficacy of treatment. (See Methods chapter 2.6.6)**.**

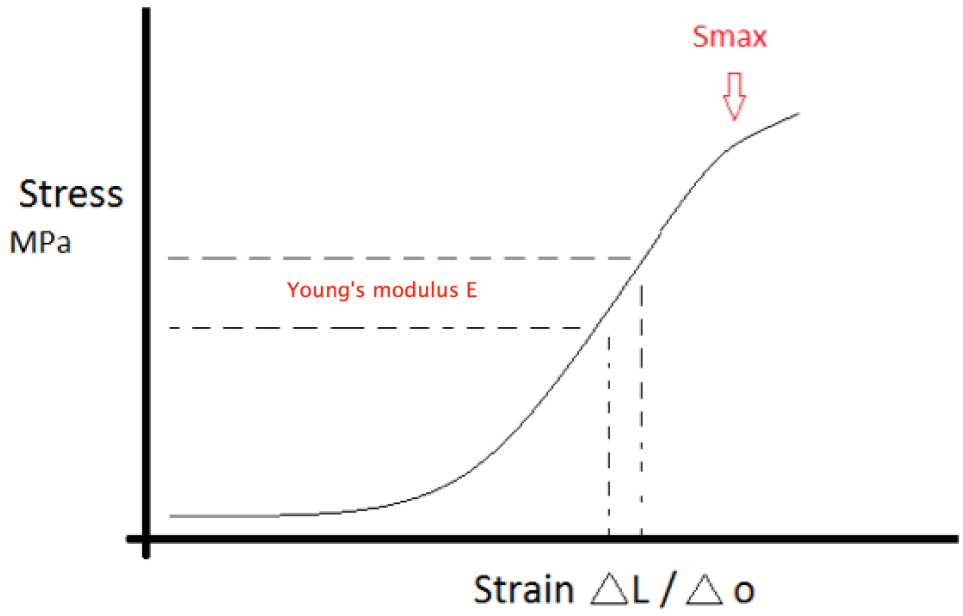


Figure 3.4. A typical stress / strain curve as obtained from the Zwick Roell tensile test. A strip of tissue is loaded into ceramic clamps and stretched at a rate of 2N/second.

Difference in Young's modulus is using two differing methods to determine if the study yields alternative results. One experiment measures the stretch force and one measures the compressive force. The two different tests give more information about the specimens. As the experiments yielded similar figures for Young's modulus, I decided to use the method which allowed the specimen to be analysed in a time efficient manner and therefore reducing effects of specimen dessciation.

3.1.3.3.1 *Young's modulus from Zwick Roell Tensile test.*

Two repeat biopsies were taken from 10 different pigs to assess the feasibility and comparability of Zwick Roell device as a mechanical test. The mean Young's modulus of repeat skin samples from the same pig was 3.12 – 4.87 +/- 0.06 -0.63. The mean Young's modulus of skin samples from different pigs was 3.99- 4.09 +/- 0.52- 0.59. The results between the different mechanical testing modalities were comparable.

Porcine Tissue	Young's Modulus MPa Sample 1	Young's Modulus MPa Sample 2	**Mean**	% Difference
1	3.11	3.20	**3.12**	2.95
2	3.94	3.82	**3.88**	3.56
3	4.01	3.89	**3.95**	3.63
4	4.12	4.21	**4.17**	3.96
5	4.21	4.42	**4.32**	4.17
6	3.98	3.81	**3.89**	3.55
7	4.41	5.02	**4.72**	4.78
8	4.76	4.98	**4.87**	4.73
9	3.13	3.43	**3.28**	3.19
10	4.25	4.07	**4.16**	3.81
Mean	**3.99**	**4.09**		
Std Dev	0.52	0.59		

Table 3.3 Young's modulus calculated from Zwick Roell

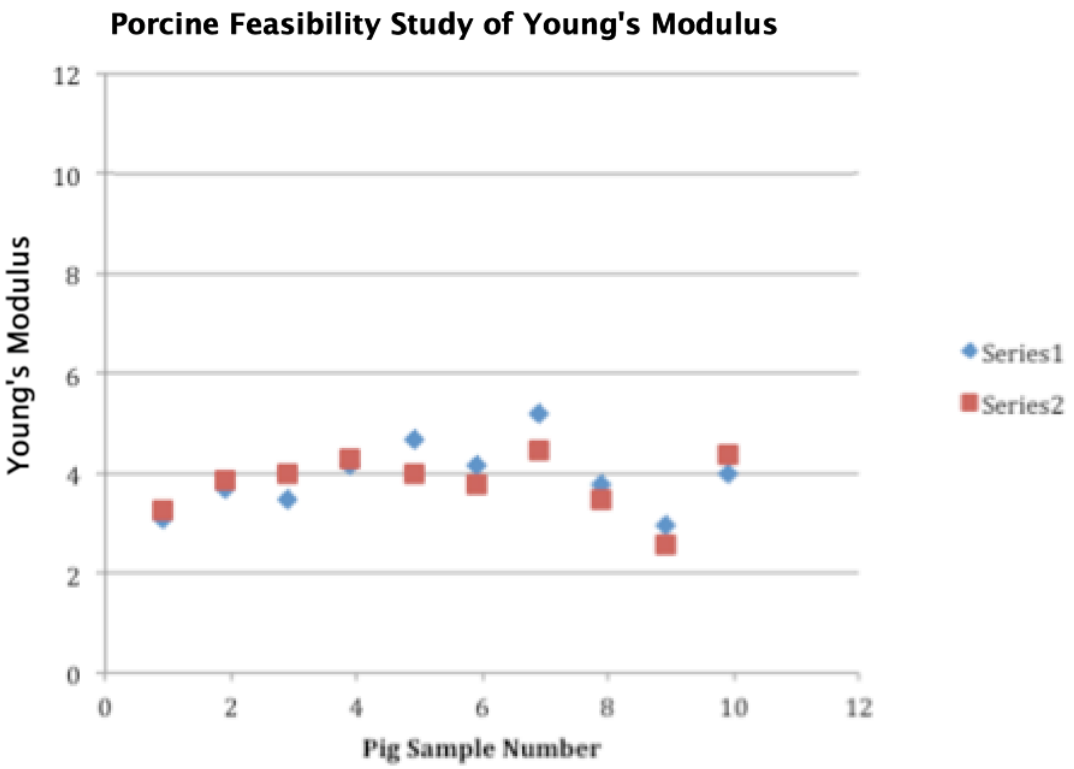


Figure 3.5. Young's modulus variation in Zwick feasibility study with different pigs

3.1.3 Discussion of Chapter 3.1 Results

The animal feasibility studies were conducted to optimize the techniques and protocols on readily available tissue. This ensured the human specimens, which were very precious, were processed appropriately.

DSC clearly showed a significant general elevation of the standardized peak heat flow in porcine samples. There was no significant inter sample variation and therefore the protocol could be applied to human studies. Collagen denaturation, which can be investigated by differential thermal analysis, is affected by non-enzymatic gyration (i.e., by additional cross-link formation). This is a method frequently used to examine

phase transitions (Le Lous et al. 1982). It involves measurement of the heat flow necessary to ensure heating of the specimen at a constant rate. Heat flow is measured against a reference specimen that does not undergo any phase transformation in the temperature range under scrutiny. During the phase transformation there is a change in the heat flow, the level of the peak heat flow and the corresponding temperature being used as parameters to characterize the phase transition (Flandin et al. 1984). An alternative method for investigating phase transitions, thermal contraction, has been used both on rat tail tendons (Le Lous et al. 1983) and on rat skin (Allain et al. 1978) . When the density of the thermostable cross-linking is elevated the maximum contraction force is also greater. Biological factors, such as species variations, are generally recognized to have impact on skin permeability. Only a few studies, have investigated the effect of species differences on the thermal behaviour of stratum corneum (Rehfeld et al. 1988). These workers showed different results, probably due to variations including heating rate, degree of hydration of samples and sampling methods. In our work, using identical experimental conditions, we investigated and compared the DSC thermograms from pigs and humans. Dry samples were used in this comparison to avoid any discrepancies that might arise due to variations in the degree of hydration of samples. In the present study, we hoped to detect variations among thermograms of the different species that may correlate with the reported differences in skin permeability among these species (Barry, 1983, Sato et al., 1991 and Walters, 1993).

Porcine skin is appropriate for in vitro studies because its topology, texture, architecture and metabolic rate are similar to those of human skin (Schmook et al. 2001). In addition, skin properties are not significantly affected by the lack of a

physiological environment provided there is significant moisture (Serup & Agner 1990). The morphology of porcine skin is similar to that of healthy human skin, even after 3 days from harvesting. Insignificant differences in the mechanical properties of the skin of different porcine breeds and randomly selected samples of each breed indicated that the measured mechanical properties were independent of breed type and order of sample testing.

Theoretical and experimental models suggest that skin tissue will yield under apparent tension than under apparent compression as a result of difference in local tissue failure modes (failure due to excessive tensile principal strains vs. compressive principal strains) (Bayraktar & Keaveny 2004), suggesting that apparent tension will result in more microdamage than apparent compression. With this and the lengthier method required to assess Young's modulus we chose to perform tensile testing.

The results demonstrate differences in Young's modulus between apparent tension and compression observed here are consistent with results from high-resolution finite element analyses that showed more tissue failure for apparent tension or shear than apparent compression loading (Bayraktar & Keaveny 2004), thereby providing support for the tissue-level asymmetric tensile-compressive principal strain failure criteria approach used in the models.

CHAPTER 4

HISTOLOGY

4.0 Histological examination of skin samples from patients and controls

4.1 Introduction

Study of the skin rather than the pelvic tissues allows us to determine if there are any systemic disorders which maybe precipitating a localised process in the aetiology of prolapse without the confounding effect of the pathology on the tissue of interest (Holbrook & Byers 1989). We examine its expression in skin, a 'window on heritable connective tissue disorders', rather than in tissue taken from the prolapse site where inherent changes can obscure pathological changes in tissue composition.

Patients with internal or external rectal prolapse suitable for surgery were recruited and consent was performed in a standard manner prior to surgery. Patients had a skin punch biopsy taken at the site of laparoscopic port insertion. (See chapter 2 figure 2.4) Controls were age and sex matched by recruiting patients from the emergency and elective surgical theatre lists and skin specimens were acquired and stored. Those who had evidence of connective tissue disease, rectal prolapse, previous pelvic surgery or active inflammatory process were excluded.

Demographic and pertinent clinical information was recorded prospectively and stored in a dedicated database.

4.2 Elastin fibres

Components of the connective tissue other than collagen have also been found to be involved in development of prolapse as elastin fibre organization differs between controls and subsets of patients with rectal prolapse (Joshi H et al 2015). Transgenic

mice have demonstrated the importance of elastic fibres for maintaining structural and functional integrity of the pelvic floor (X. Liu et al. 2006; Rahn et al. 2008; McLaughlin et al. 2006; Drewes et al. 2007).

Elastin fibres consist of a central elastin core, surrounded by a mantle of microfibrils. Their synthesis and assembly is a complex process involving a number of different proteins such as fibrillin-1 and fibulin-5 integral in this process (Schiemann et al. 2002). Mutations are involved in various disorders such as neuropathies, macular degeneration and in cutis laxa, a disease characterized by loose, redundant skin (Stone et al. 2004; Elahi et al. 2006). Case reports have described prolapse in a subpopulation of women with cutis laxa (Hu et al. 2006); also mice with null mutation in fibulin-5 (*Fbln5*$^{i/i}$) develop a prolapse that is remarkably similar to that of primates (X. Liu et al. 2006; Rahn et al. 2008; McLaughlin et al. 2006).

4.2.1 Measurement of elastin fibres

Elastin fibre composition was performed by undertaking histological testing (see chapter 2.6.2) and slides were viewed using a light microscope. Digitised images were captured using a microscope-mounted camera. These images were saved as high-resolution tiff files. This image was then uploaded onto an Image analysis software programme (Image J, obtained from: Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). The image threshold was adjusted such that only the darkly stained elastic fibres were visualised. The area occupied by these fibres was then calculated electronically. Two independent, blinded observers scored scores.

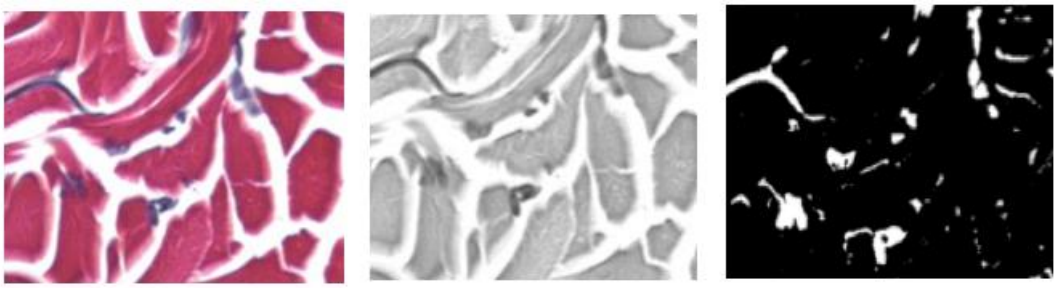


Figure Image J analysis

Figure 4.1: The percentage of dermal elastin fibres in skin samples from patients with internal prolapse and external prolapse. Prolapse scored according to (Harmston et al. 2011)

4.2.1.1 Dermal elastin fibres in control patients

In our control samples, elastin fibre were found to occupy 5.0% (3.88-6.56 +/-0.90) of the total surface area. These figures are in agreement with findings in the literature [15]. The fibres typically had a mature morphology and were aligned to collagen fibres in the dermis (figure 5.2). Measurement of fibres as described above in 4.2.1

4.2.1.2 Dermal elastin fibres in prolapse patients

The patients with rectal prolapse had a greater proportion of dermal elastin fibres than those without prolapse. This was most marked in males suffering from prolapse who had 8.3% (range 6.8-13.2 +/- 2.5) surface area occupied by elastic fibres, an increase of 4.3% (p= 0.001). Comparison between the groups suffering from prolapse did not show any significant difference between groups. Although these differences in density of fibres between patients and controls were identified, the elastin fibre themselves appeared morphologically similar between prolapse and control group.

(a) (b)

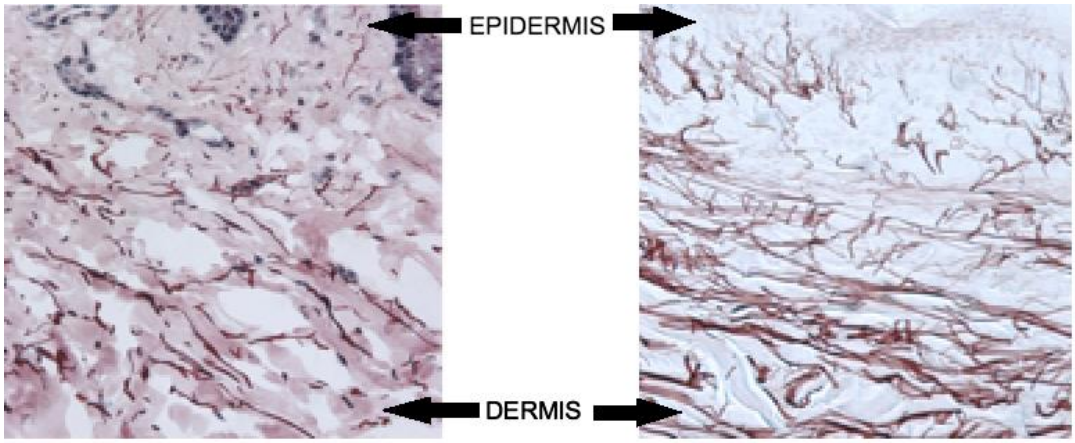


Figure 4.2: Typical results of elastin organization in skin samples stained with Orcein and observed under light microscope (a) Control sample skin, (b) Prolapse skin. (Age matched specimens)

Orcein stain (x20 magnification)

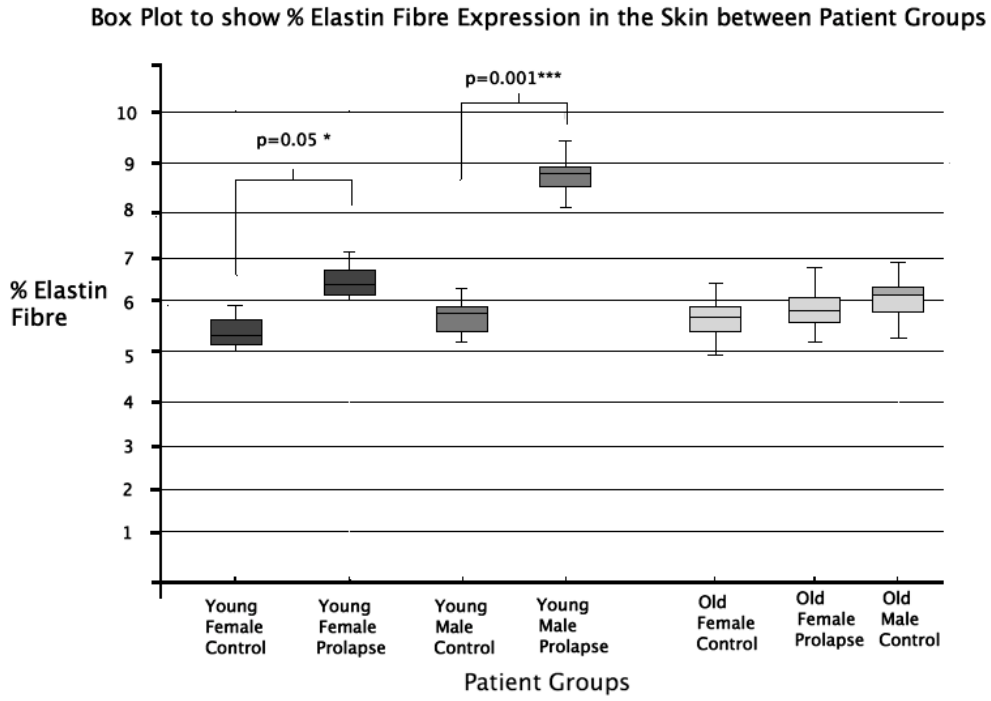


Figure 4.3. – Box plot to show elastin fibre expression in the skin of age and sex matched prolapse patients against controls.

4.2.1.3 Dermal elastin fibres and prolapse severity

We examined density of elastin fibres in relation to prolapse severity and compared young (28-43 yrs) and old (67-82years) patients at each severity grade. The data demonstrated changes in the proportion of elastin fibres with a trend towards increased elastin expression in those suffering from prolapse. Individuals were compared according to stage of prolapse (internal vs. external).

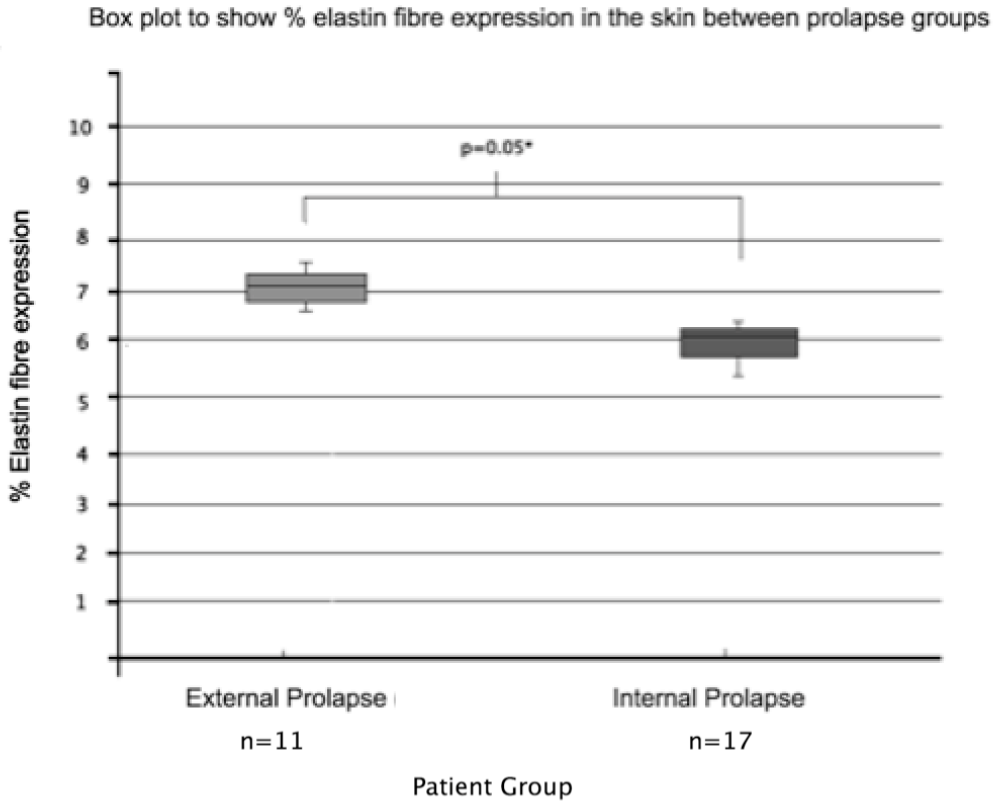


Figure 4.4: Box plot to show elastin fibre expression in the skin of patients with external prolapse and internal prolapse

It was found that in patients with external prolapse, 8.2+/- 5.95% (range 6.44-19.11) of the surface area was occupied by elastin fibres, compared with individuals with internal prolapse who had 6.9+/- 3.11% (range 3.29- 12.97) surface area occupied by elastic fibres (Figure 4.4). These differences were significant (p= 0.05).

4.2.1.4 Elastin expression in relation to connective tissue status

The expression of elastin was similar between patients in relation to connective tissue status. Patients with features of BJH had a slightly higher percentage expression 7.6% (7.1-7.9 +/- 0.20) compared with the normal groups of 7.4% (7.3 -7.9 +/- 0.14).

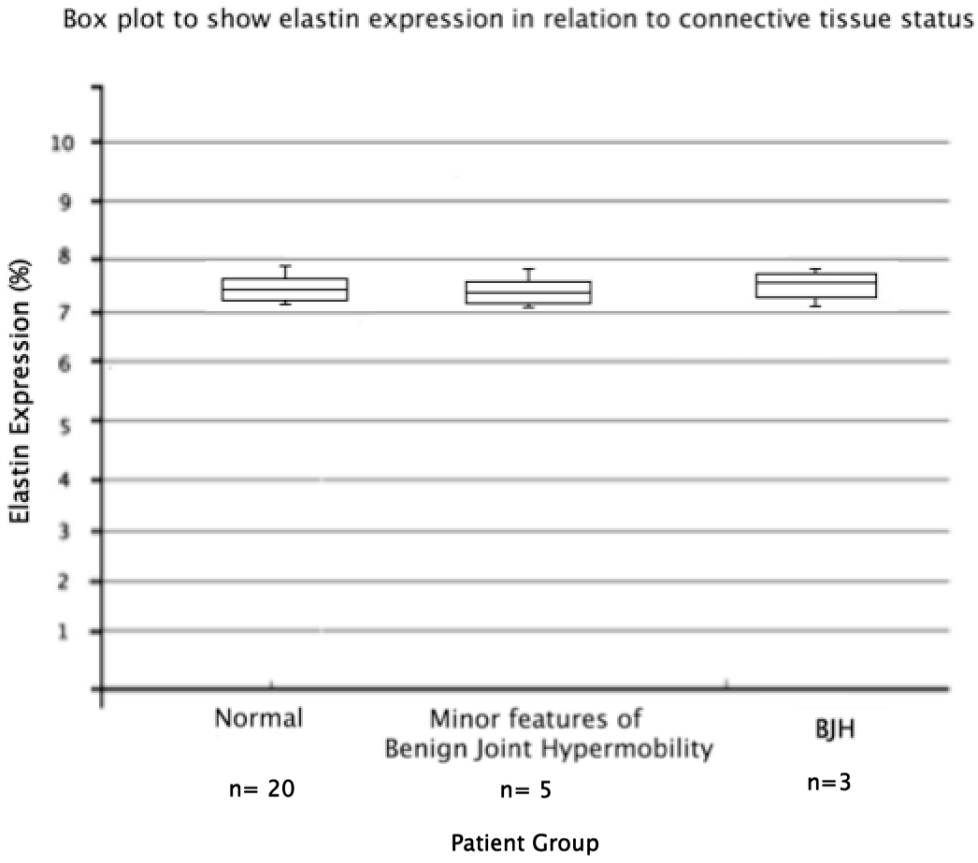


Figure 4.5. Box plot to show elastin expression in relation to connective tissue status

4.3 Fibrillin in skin samples from controls and patients with prolapse

Fibrillin-1 is a large, extracellular matrix glycoprotein that serves as a structural component of 10-12 nm calcium-binding microfibrils. These microfibrils provide force bearing structural support in elastic and nonelastic connective tissue throughout the body. Mutations in this gene are associated with Marfan syndrome (Freeman et al. 2005). Mice with null mutations in genes involved in elastin synthesis develop spontaneous prolapse (Budatha et al. 2011). We wanted to assess whether fibrillin-1 was differentially expressed in the skin of age, sex matched patients and controls.

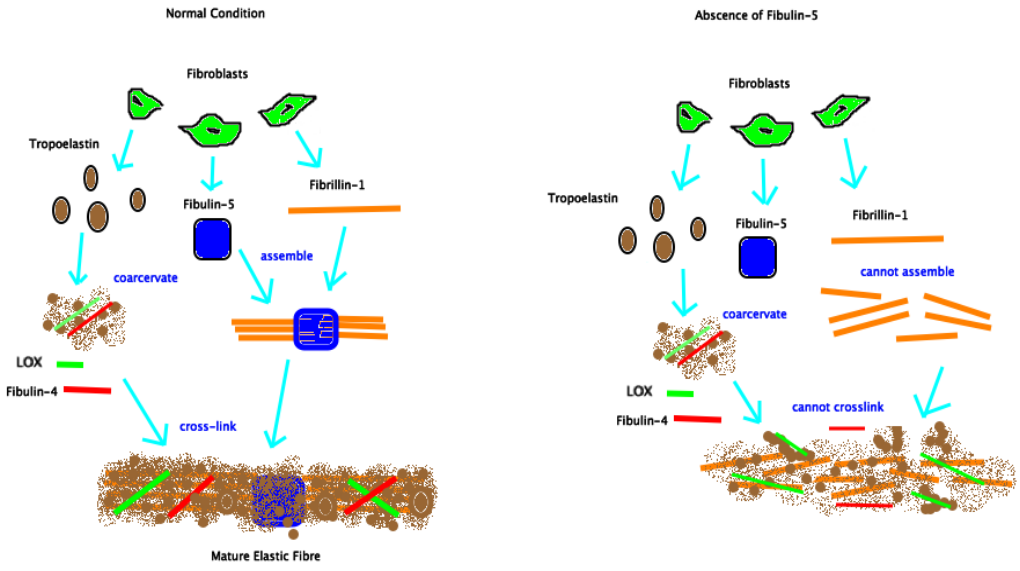


Figure 4.6 Schematic diagram showing the role-played by fibrillin-1 and fibulin 5 in elastin fibre synthesis

4.3.1 Fibrillin- 1 in control patients

In our control samples, fibrillin-1 was found to occupy 5.8% (4.98-6.21 +/-0.90) of the total surface area. These figures are in agreement with similar findings in the literature (Carta et al. 2006) The fibres typically were aligned to collagen fibres in the dermis (Figure 5.6).

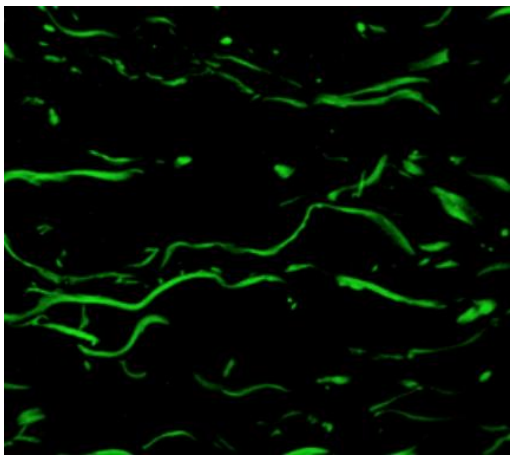


Figure 4.7. Immunohistochemistry slide at 20x magnification of Fibrillin-1 in human skin

4.3.2 Fibrillin-1 in prolapse patients

The patients with rectal prolapse had a similar proportion of fibrillin-1 than those without prolapse. Comparison between the groups suffering from prolapse did not show any significant difference. The fibrillin-1 expression appeared morphologically similar between prolapse and control groups.

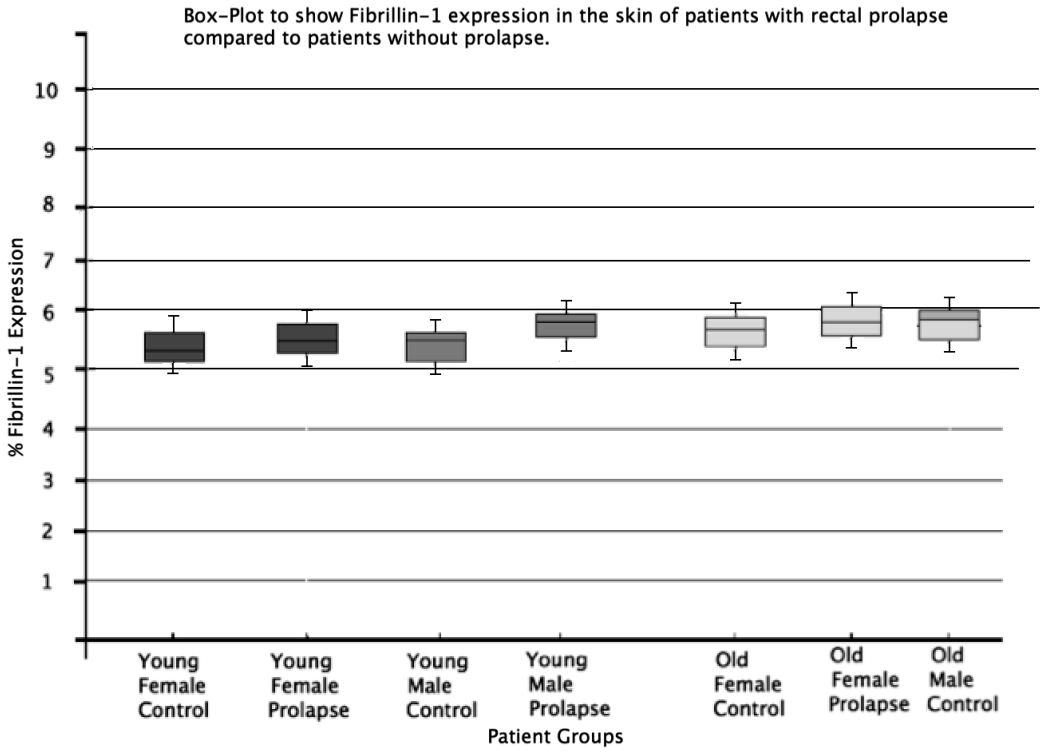


Figure 4.8. Box plot to show percentage fibrillin-1 expression in the skin of patients with rectal prolapse compared to patients without rectal prolapse.

4.3.3 Fibrillin-1 and prolapse severity

The expression of fibrillin-1 was similar between patients with varying severity of prolapse. Patients with external prolapse the more severe form of the disease had a slightly lower percentage expression 5.5% (5.2-5.9 +/- 0.30) compared with the less severe prolapse groups of 5.8% (5.4 -5.9 +/- 0.12).

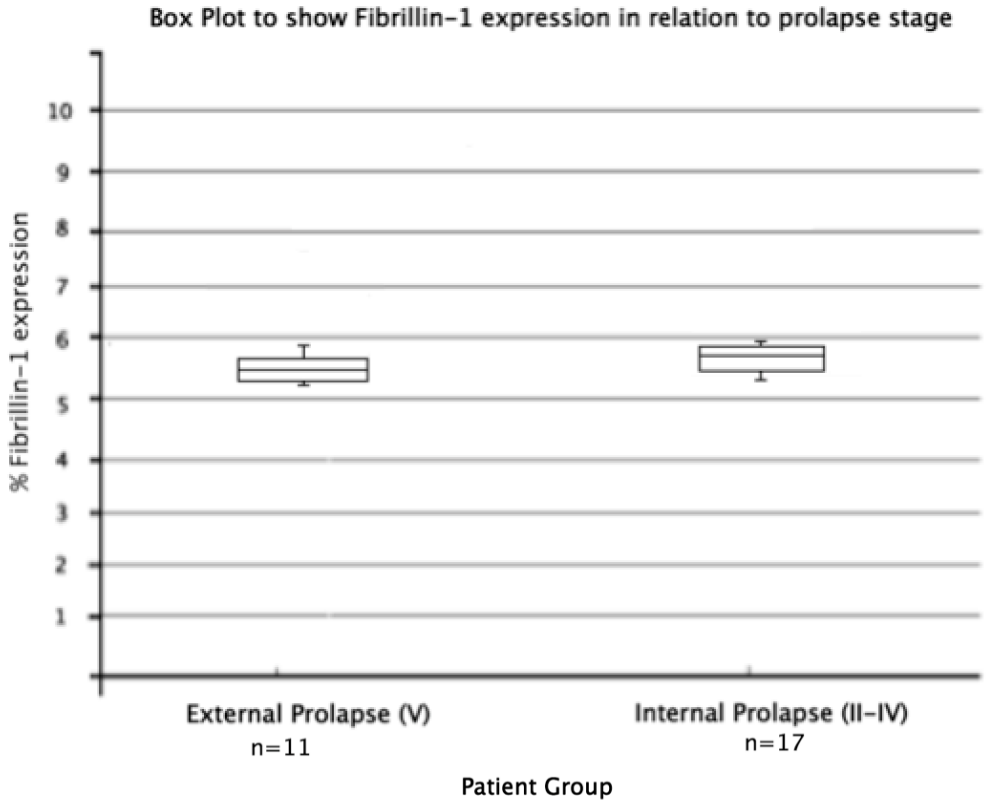


Figure 4.9. Fibrillin -1 expression in relation to prolapse severity

4.3.4 Fibrillin-1 expression in relation to connective tissue status

The expression of fibrillin-1 was similar between patients in relation to connective tissue status. Patients with features of BJH had a slightly higher percentage expression 5.8% (5.1-5.9 +/- 0.21) compared with the normal groups of 5.6% (5.3 - 5.9 +/- 0.14).

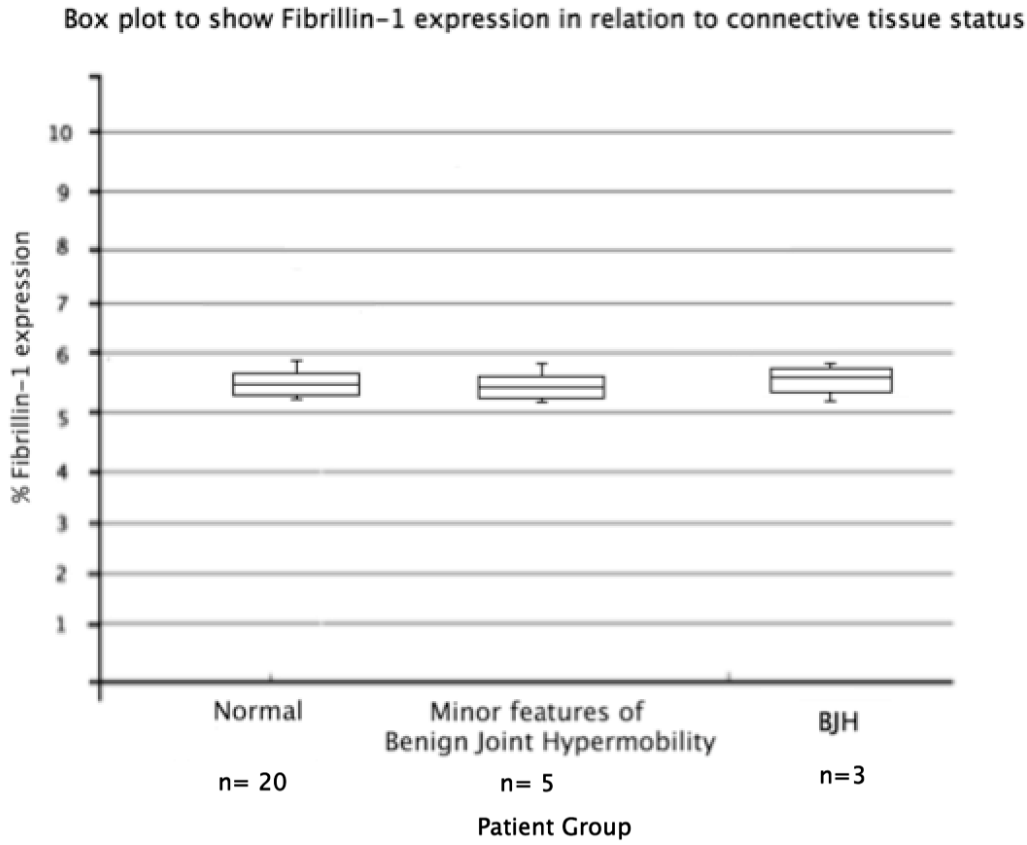


Figure 4.9a Box plot to show fibrillin-1 expression in relation to connective tissue status.

4.4 Fibulin 5 in skin samples of patients and controls

Elastin fibre synthesis and assembly is a complex process involving a number of different proteins with the fibulin-5 integral in this process (Schiemann et al. 2002). Fibulin-5 is a multi- functional extracellular-matrix protein that regulates cell growth, motility, and adhesion as well as elastogenesis (Sultan 2009). Mutations are involved in various disorders such as neuropathies, macular degeneration and in cutis laxa, a disease characterized by loose, redundant skin (Stone et al. 2004; Elahi et al. 2006). Moreover, case reports have described prolapse in a subpopulation of women with cutis laxa (Markova et al. 2003; Paladini et al. 2007); also mice with null mutation in fibulin-5 (*Fbln5*¡/¡) develop a prolapse that is remarkably similar to that of primates (Liu et al. 2004; Wieslander et al. 2009; McLaughlin et al. 2006).

We examined the pattern of FIB-5 immunostaining and the staining intensity in patients and controls in relation to age, gender, prolapse severity and hypermobility status. The staining intensity is calculated by the mean intensity scores from two blinded observers (see methods 2.6.1)

4.4.1. Fibulin 5 expression in control patients

In our control samples, immunostaining was localized to the extra-cellular matrix with a fine and even distribution. The median intensity score for control patients was 2 (range 0-3). (Figure 4.9b and 4.9c).

4.4.2 Fibulin 5 expression in prolapse patients

The immunostaining for FIB-5 was localized to the ECM with a fine and even distribution among the samples. There was no difference in staining intensity between the skin from women with or without prolapse [Intensity score, median (range), 2.0 (0–3) vs. 2.0 (0–3), NS]. The staining intensity was significantly decreased in the skin of men with prolapse compared to men without rectal prolapse. Both intracellular and extracellular FIB-5 immunostaining was found [Intensity score, median (range), 1 (0–3) vs. 2 (0– 3), $P = 0.05$] (Figure 4.9b and 4.9c)

To evaluate inter-observer agreement, κ for each pair of observers was calculated. Kappa and κ′ for multiple observers was calculated for each scoring category. Kappa and κ′ values were interpreted using the following guidelines: poor agreement <0.20, fair agreement 0.21−0.40, moderate agreement 0.41−0.60, good agreement 0.61−0.80, and very good agreement 0.81−1.00. (Cohen et al.1968).

The median (minimum−maximum) κ for each pair was 0.80 (0.78−0.85). Multiuser κ (95% CI) for assessment of fibulin-5 expression was 0.79 (0.77−0.81), and multiuser κ′ was 0.77 (0.75−0.81).

4.4.3 Comparison of FIB-5 expression between patients and controls

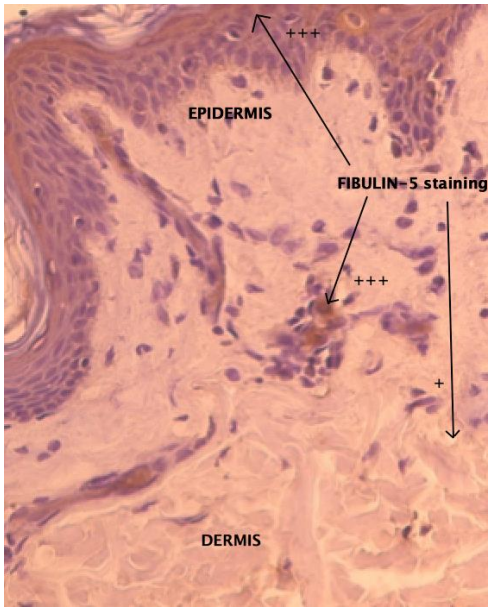


Figure 4.9b. Human skin sample Fibulin staining scoring intensity.



Figure 4.9c. Human skin sample. No fibulin-5 expression.

Table 4.1. FIB-5 intensity score in young and old prolapse patients and controls showing the number of patients at each intensity score.

	FIBULIN -5			
	0 (-)	**1 (+)**	**2 (++)**	**3 (+++)**
Prolapse Group	**4**	**22**	**3**	**1**
Young Prolapse	4	14	0	0
Old Prolapse	0	8	3	1
Control Group	**2**	**16**	**10**	**2**
Young Control	1	8	6	1
Old Control	1	8	4	1

There was no difference in staining intensity between the skin from women with or without prolapse [Intensity score, median (range), 2.0 (0–3) vs. 2.0 (0–3), NS]. The staining intensity was significantly decreased in the skin of men with prolapse compared to men without rectal prolapse

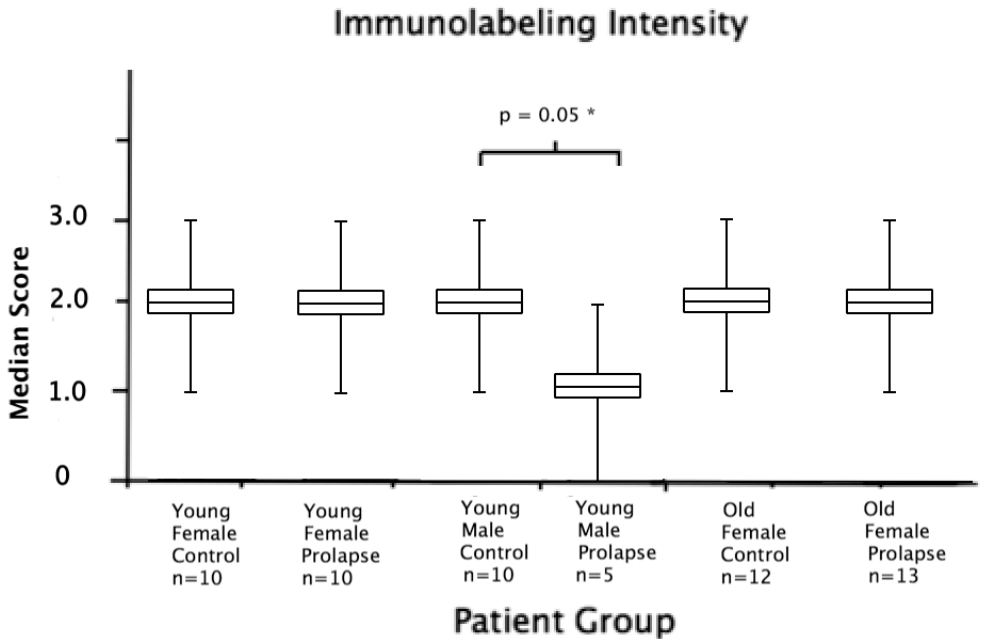


Figure 4.9d. FIB-5 immunostaining intensity score in patients and controls in relation to age and gender. amongst patient groups with rectal prolapse and without.

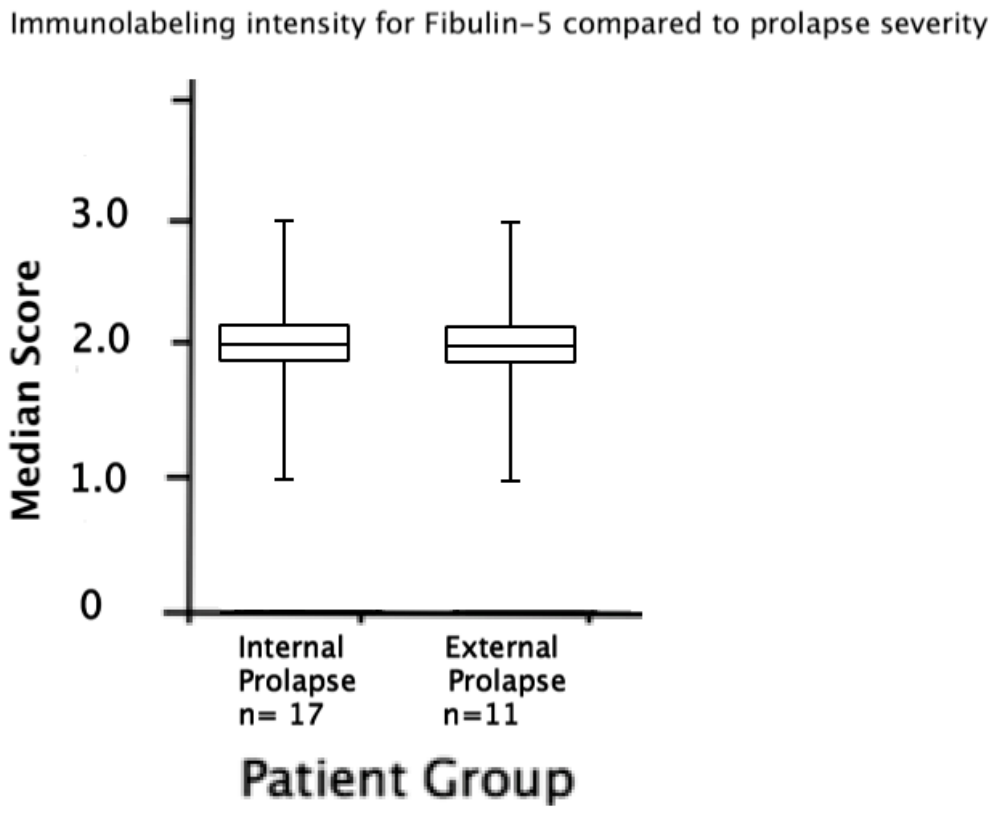


Figure 4.9e Immunolabeling intensity of fibulin 5 compared with prolapse severity.

4.4.3 Fibulin 5 expression according to severity of prolapse grade

There was no difference in staining intensity between the skin from patients with internal and external prolapse. [Intensity score, median (range), 1.75 (0–3) vs. 1.75 (0–3), NS].

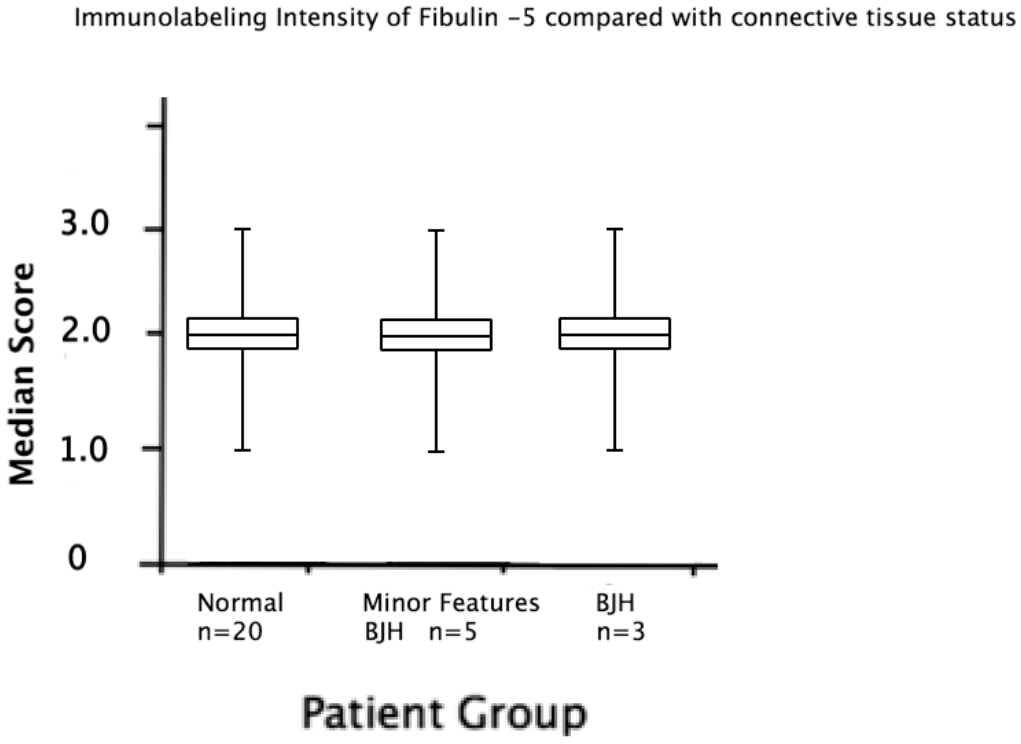


Figure 4.9f. Fibulin-5 expression compared to patient hypermobility status.

4.4.5 Fibulin 5 expression according to patient hypermobility status

There was a difference in staining intensity between the skin from patients with no clinical evidence of joint hypermobility and patients with features of joint hypermobility. [Intensity score, median (range), 2.0 (0–3) vs. 2.0 (0–3), NS].

4.5. Discussion

4.5.1 Summary of results of histological staining of patient and control skin.

Younger females and males had a significantly higher percentage of dermal elastin fibres and patients with external prolapse had a higher percentage of dermal elastin fibres. There was an increase in dermal elastin fibres in patients with features of joint hypermobility but this was not significant.

There was no statistical difference in fibrillin-1 expression amongst age/sex matched patients and controls or when comparing prolapse severity or connective tissue status. When assessing fibulin-5, there was no difference in staining intensity between the skin from women with or without prolapse. The staining intensity was significantly decreased in the skin of men with prolapse compared to men without rectal prolapse. There was no statistical difference in fibulin-5 expression amongst age/sex matched patients and controls or when comparing prolapse severity or connective tissue status.

4.5.2 Changes in elastic fibre components in patients relative to controls

Study of the skin rather than the pelvic tissues allows us to determine if there are any systemic disorders which maybe precipitating a localised process in the aetiology of prolapse without the confounding effect of the pathology on the tissue of interest (Holbrook & Byers 1989). The results demonstrate an increased density of elastin fibre in patients with rectal prolapse. The controls correlate with previous work

undertaken in the dermatological setting (Langton et al. 2010). The study data provides further suggestion of the existence of an occult connective tissue disorder in individuals suffering from rectal prolapse. The extent to which defects in elastic fibre synthesis or function are responsible is unclear at the present time. However, animal models with defects in elastin fibre synthesis are more prone to pelvic organ prolapse (Zheng et al. 2006; Karnik 2003).

To date, the limited work that has been undertaken in relation to rectal prolapse, has tended to focus on the potential for a collagenous disorder underpinning the disease (Jackson et al. 1996; Willis et al. 2010), whilst this study by no means contradicts those findings, it does suggest that in most cases the aetiology of rectal prolapse is likely to be multifactorial. Indeed, the presence of increased elasticity may exacerbate any existing underlying collagenous predisposition to prolapse. The experimental findings correlate with previous published work describing the presence of elastic fibres in the rectal mucosa of patients suffering from rectal prolapse (Warren et al. 1990). The elastic fibres were composed of individual globular units of elastin integrated within the microfibril bundles (Figure 6.0). The elastin component of the elastic fibre was always observed in close proximity to the microfibril bundle despite the absence of fibulin-5. Thus, although fibulin-5 binds both tropoelastin (Yanagisawa et al. 2002) and fibrillin-1 (Freeman et al. 2005; El-Hallous et al. 2007; Kobayashi et al. 2007), this molecular interaction is not required for the co-localization of these two major elastic fibre components in the dermis.

Fibulin-5 is required to mediate the appropriate integration of elastin into the microfibril bundle. This role of fibulin-5 could either be by simply restricting the size

of the elastin globules so that they can become incorporated into the core of the microfibril bundle or by more directly aiding in the transfer and/or linking of tropoelastin monomers onto the fibrillin-1 microfibrils at a molecular level. Finally, because of the regional disparity observed with respect to the size of the elastin globules, there must be some additional protein(s) or mechanism that is responsible for this difference in elastin composition (Papke & Yanagisawa 2014).

CHAPTER 5

MECHANICAL TESTING

5.0 Mechanical properties of human skin

Many surgical subspecialities recognize the benefits of rigorous biomechanics research to potentially improve patient care, however many of the studies focus on analysis of the diseased tissue, in our study we examined the skin from an unaffected area to see if there are intrinsic differences between patients who suffer with prolapse and controls. A tissue's ability to either generate or transfer forces is inherently coupled to its biochemical composition and spatial organization. Consequently, a deeper understanding of the biomechanics of the pelvic floor has the potential to shed new light on the mechanisms of pelvic floor disorders and our potential to treat them (Goh 2003).

5.1 Differential scanning calorimetry- Human Skin

Patient Group	Number	Mean age (range) yrs.
Young female control	10	33 (29-35)
Young female prolapse	10	32 (30-32)
Young male control	10	35 (28-43)
Young male prolapse	5	33 (30-36)
Old female control	12	74 (71-82)
Old female prolapse	13	79 (77-82)
Old male control	10	72 (67-82)

Table 5.1: Numbers of patient and control samples used in DSC testing.

5.1.1 Enthalpy changes in human skin samples

5.1.1.1 Enthalpy in control patients

In our control skin samples, enthalpy measured by DCC (see section 2.7.1) was found to be 9.7 +/-0.76) (6.9-14.8) in females and 9.8+/-0.92 (6.25 -17.35) in males.

5.1.1.2 Enthalpy in prolapse patients

The skin samples from patients with rectal prolapse had a similar enthalpy to those without prolapse. This was the case for both sexes with females having an enthalpy of 9.8+/- 0.27 (6.7-14.7) and males having 9.9+/- 0.82 (range 6.6-18.4). The difference in results did not reach statistical significance however.

Results for the different groups of patients and controls are shown below. While there was no statistical difference between groups, it can be seen that the enthalpy of older patients and controls tended to be greater than that of the younger patients and controls, and also that the results for patients, especially young patients, were far more variable than those of the controls.

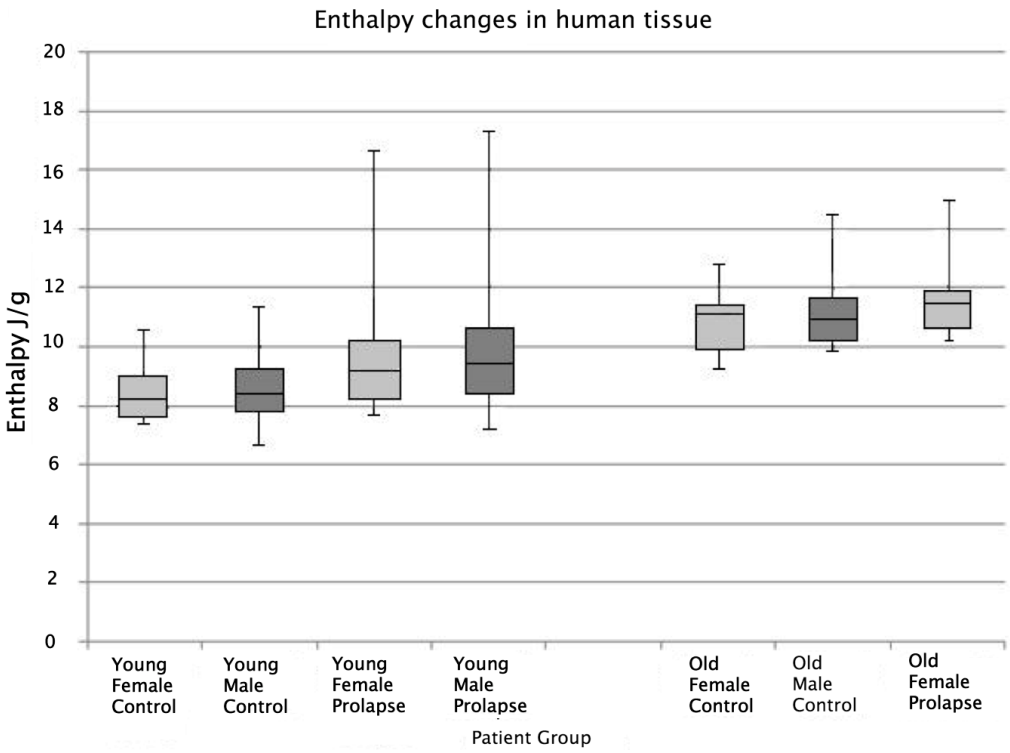


Figure 5.1. Box plot to show enthalpy changes in human skin samples of patients with prolapse and controls.

5.2 Dynamic Mechanical Analysis –Human Skin

5.2.1 Young's Modulus in control patients

In our control samples, Young's modulus measured by dynamic mechanical analysis (Section 2.7.2) was found to be 2.4 +/-0.30 (1.9-2.8) in females and 3.15+/-0.20 (2.25 -3.35) in males.

5.2.2 Young's Modulus in prolapse patients

The young patients with rectal prolapse had a lower Young's modulus than those without prolapse but for the old females, controls and prolapse showed similar values This was the case for both sexes with females having a lower Young's modulus of 2.2+/- 0.2 (2.5-2.8) and than the males having 2.9 4 +/- 0.2(range 2.6-3.). The difference in results did not reach statistical significance however.

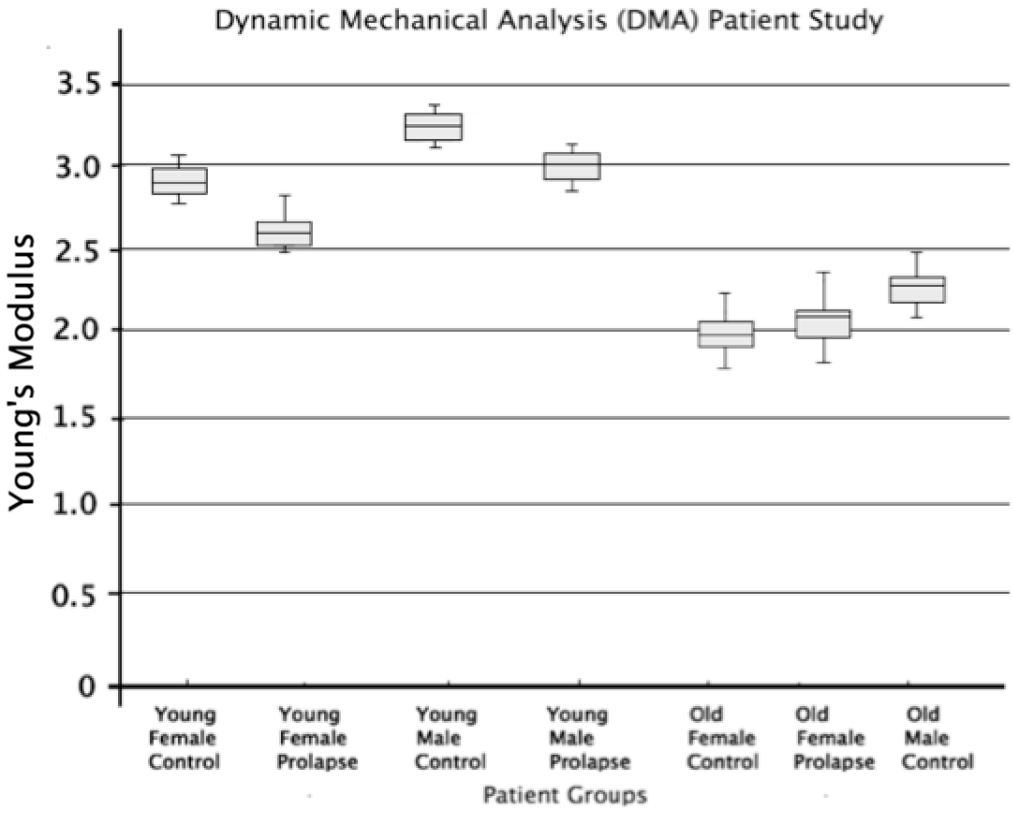


Figure 5.2. Box plot showing Young's modulus measured by dynamic mechanical analysis of skin samples patients with prolapse compared to patients without.

5.3 Tensile Testing Zwick Roell – Human skin

Patient Group	Number	Mean age (range) yrs.
Young female control	12	33 (29-35)
Young female prolapse	11	32 (30-32)
Young male control	11	35 (28-43)
Young male prolapse	7	33 (30-36)
Old female control	14	74 (71-82)
Old female prolapse	14	79 (77-82)
Old male control	12	72 (67-82)

Table 5.2. Number of patients used for Zwick Roell testing

5.3.1 Young's Modulus of the skin in control patients

In our control samples, Young's modulus was found to be 3.2 +/-0.20 (3.1-3.3) in females and 3.3 +/-0.20 (3.2 -3.4) in males.

5.3.2 Young's Modulus of the skin in prolapse patients

The patients with rectal prolapse had a lower Young's modulus than those without prolapse. This was the case for both sexes with females having 2.6 (2.5-2.8 +/- 0.2)

and males having 2.9 (range 2.6-2.9 +/- 0.2). The results were statistically significant in both groups.

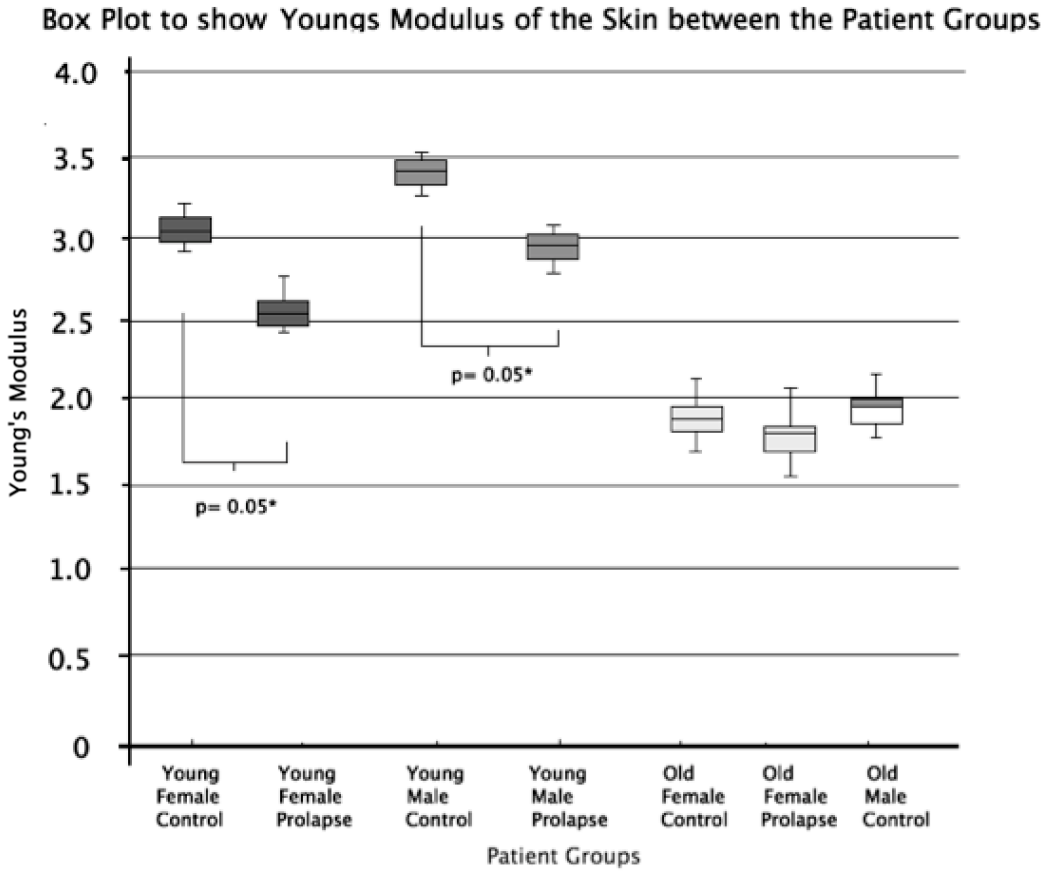


Figure 5.3: Young's modulus from mechanical tensile testing of skin samples from prolapse and control patients.

The values of Young's modulus of skin samples measured using the Zwick Roell were similar in pattern to those measured using DMA; the ratio is seen in Table 5.3

Table 5.3. Comparison of measurements of Young's modulus of skin samples from patients and controls between those made with DMA and those made with Zwick Ratio.

Subject	DMA MPa (mean)	Zwick MPa (mean)	Ratio
Young female control	2.9	3.2	0.91
Young female prolapse	2.6	2.6	1.00
Young male control	3.2	3.3	0.97
Young male prolapse	2.8	2.9	0.96
Old female control	1.9	1.8	1.05
Old female prolapse	2.0	1.7	1.17
Old male control	1.9	2.2	0.86

5.3.3 Young's Modulus of the skin as a function of connective tissue status

Young's modulus was lower in patients with clinical evidence of benign joint hypermobility (measured using Beighton score – Table 2.5), compared with those patients who had no evidence of benign joint hypermobility or normal connective tissue status. (Numbers of patients shown in table 5.2). Although the numbers of patients with joint hypermobility was low, there was a statistical difference between normal patients 3.2 (range 2.9-3.4 +/- 0.2) and patients with features of BJH 2.6 (range 2.4-2.8 +/- 0.2)

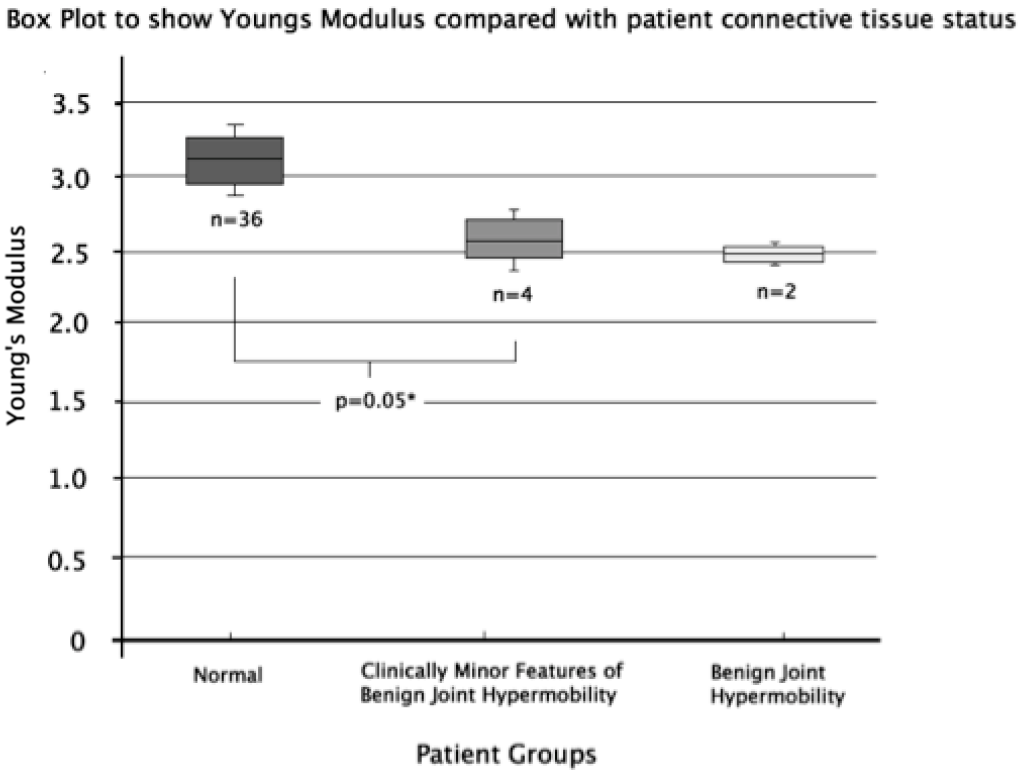


Figure 5.4: Young's modulus of skin samples in patients measured Zwick Roell tensile testing of patients compared with connective tissue status.

5.3.4 Young's Modulus of skin compared with severity of prolapse

The data demonstrated a trend towards increased Young's Modulus of the skin in those suffering from less severe prolapse. Individuals were compared according to stage of prolapse (internal vs. external). It was found that individuals with internal prolapse had a Young's Modulus of 2.7 MPa (2.6-2.9 +/-0.25) compared with individuals with internal prolapse who had a Young's modulus of 2.6MPa (2.5- 2.9 +/- 0.26) (Figure 5.5). These differences were not statistically significant.

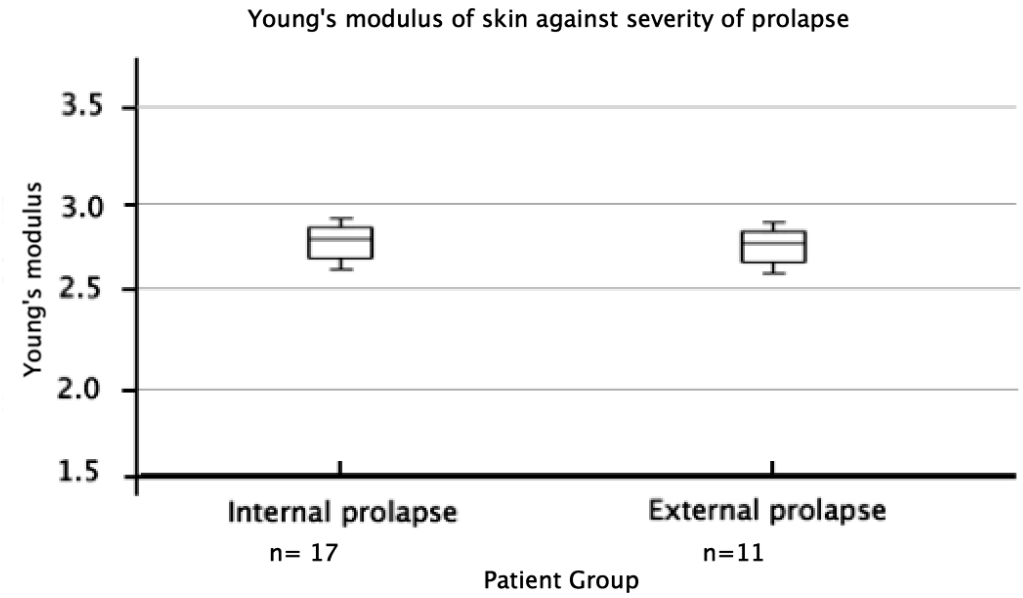


Figure 5.5 Young's Modulus as a function of the severity of prolapse and stage.

5.4 Human pelvic connective tissue - Tensile Testing using the Zwick Roell –

Patient Group	Number	Mean Age (range) yrs.
Young female prolapse	6	33 (29 -35)
Young male prolapse	5	32 (30 -32)
Old female prolapse	11	72 (67 -82)
Old female control	6	74 (68 -85)

Table 5.4. Number of patients used in tensile testing of pelvic tissue.

5.4.1 Young's Modulus of pelvic connective tissue in control patients

In our female control samples, Young's modulus was found to be 7.4MPa (7.1-8.1 +/- 0.20).

5.4.2 Young's Modulus of pelvic connective tissue in prolapse patients

The patients with rectal prolapse had a greater Young's Modulus than those without prolapse. This was the case for both sexes with females, having a Young's Modulus of 10.2MPa (9.8-11.7 +/- 0.22) and males having a Young's Modulus of 10.4MPa (range 9.9-11.9 +/- 0.21). The results were statistically significant for all groups.

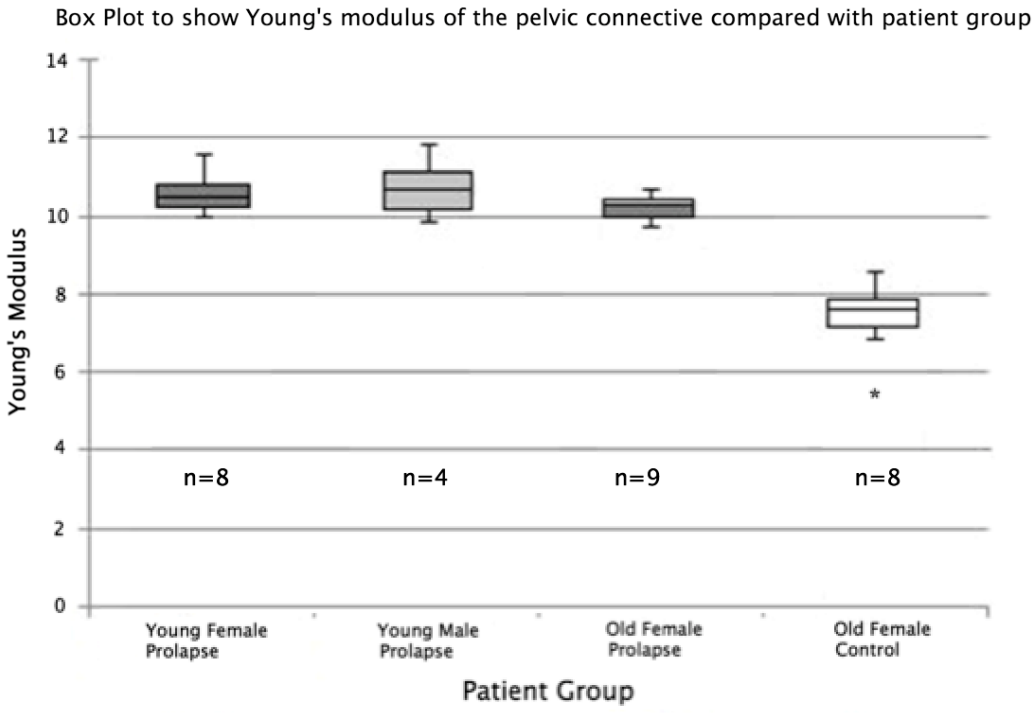


Figure 5.6. Box plot to show Young's modulus of the pelvic connective tissue compared with patient group.

5.4 Discussion

5.5.1 Differential scanning calorimetry in human skin

DSC analysis of skin samples of human abdomen showed three major thermal transitions, T1, T2 and T3 that occurred at 38– 46, 67–73 and 76–87°C, respectively (Figure 2.7). Similar transition values have been previously reported (Al-Saidan et al. 1998) and in the present work, a weak transition at 56°C was also observed with the animal and human abdominal samples. The intensity of this transition varied by sex and pathology.

In their DSC studies of human skin, Silva et al reported only two lipid transitions, at 44°C and 68°C, from human skin. However, comparison between their work and the present study is difficult. Silva et al. used hydrated samples (40%), prepared skin membranes by a heat separation (Silva et al. 2006). Variations in skin permeability among species have also been attributed to several factors, such as degree of hairiness, variability of growth pattern, differences in sebaceous mechanisms, sweating and tissue layer thickness (McCreesh, 1965).

Variations in skin permeability have also been attributed to the quantity of intercellular lipids (Elias et al., 1983; Lampe et al., 1983), to the quality and quantity of stratum corneum lipids (Smith et al., 1982), or to the amount of skin surface lipids (Sato et al., 1991). Nevertheless, all these authors have studied the effect of species variations from a particular angle, presenting reasonable justifications for their explanations. These explanations may, however, not necessarily hold for a wider range of penetrants or animal species. Hence, the effect of species variations on skin permeability requires further investigation using different techniques concurrently, such as thermal analysis, lipid analysis and permeability studies.

5.5.2 Mechanical testing of human skin

Our data illustrates the nonlinear characteristics of human skin. For small displacements the torque output appeared linear to input, being of the same sinusoidal form as the input. Displacements above this level demonstrated the typical mechanical response of skin- a relatively low dynamic stiffness at small amplitudes rapidly increasing in stiffness as the displacement increased (Tong & Fung 1976).

Figure 3.2 shows how the torque response doubled in magnitude as the displacement was increased by one-third.

Whenever such non-linear problems are encountered in engineering problems, the general practice is to conduct small amplitude tests about fixed amplitude levels. In this manner a complete picture of the non-linear characteristics can be built up on the basis of linear results (Ridge & Wright 1966). A typical test result obtained when applying this technique to the analysis of human skin is shown at the bottom of Figure 3.3. Using a sinusoidal displacement applied at a steady level an almost linear torque response was obtained. It is, however, still apparent that the curve demonstrates a greater stiffness at the higher displacement. If the problem were no more complex than this, then an analysis of the visco-elastic properties based on such small amplitude tests would be quite valid. Due to the ability of the dermis to re-orient itself under the action of a load, it is possible to obtain completely different results from what appears to be exactly the same test (Gennisson et al. 2010).

In practice, a trapezoidal input waveform has been found useful in determining the clinically significant characteristics of the mechanical response of skin over the whole physiological range. A typical test sequence for such a trapezoidal (quasi-static) test is given in Figure 3.2. In the sequence illustrated, a displacement was uniformly applied over a period, -in this case 30 seconds. During a 30 second period, the displacement was held constant before finally being reduced to 0” over the period. This nonlinear behaviour of the skin is now a well-reported test result. Ridge and Wright (1965) have reported a similar load time relationship for quasi-static uniaxial tests on human skin in vitro (Ridge & Wright 1966). A knowledge of skin structure, permits a prediction

of the behaviour of the dermis under load. Distortion of the fibre-fluid network of the dermis inevitably causes a certain degree of fluid shift within the test area. Such distortion could cause the displacement of fluid to the surrounding test area as causing accelerated drainage of the fluid by the lymph capillaries. This change in fluid content could then give rise to the difference in peak values between the first and subsequent runs (Khatyr et al. 2004).

Although a certain degree of fluid shift must take place during these deformations. It is proposed that straining of the human dermis causes a residual orientation of some of the dermal fibres, even though the points of application of the strain are returned to their original position (Khatyr et al. 2004). Complementary mechanical studies have indicated that such a fibre rearrangement is dependent on the magnitude of the strain applied (Pailler-Mattei et al. 2008).

Whilst very small deformations can be assumed to be characterised by linear coefficients, skin displacements experienced under normal physiological conditions are far from such simple characterisations (Edsberg et al. 1999). Using the system described, a complete picture of the mechanical behaviour of skin is being developed gradually on the results of small amplitude step tests over the whole physiological range. It is hoped that from the basis of a wide variety of clinical studies a better understanding of the dynamic properties of skin can be obtained for both high and low strain rate conditions.

A systematic variation with site was also apparent in the slope of the terminal, stiffest, phase of the load-extension response. The results obtained with different test

orientations were similar, although the magnitude of the initial, high compliance, extensions are different, reflecting the anisotropy of the skin (Pailler-Mattei et al. 2008).

Excised parallel-sided skin strips used for uniaxial tension tests in vitro have defined; stress free, lateral boundaries and homogeneous deformations are present. Attempts to reproduce uniaxial tension test on in vivo skin produce inhomogeneous strain fields, with a large part of the deformed area being under a system of complex strains. It is also impossible to define the loads, or stresses, acting on specific sites within the overall test zone. The local strains can, however, be obtained as a function of the load intensity applied by the loading tabs (Kvistedal & Nielsen 2009). These representations are qualitatively similar to the response of skin to in vitro uniaxial tension tests; and they may be divided into the typical three phases of response - an initial compliant phase when large extensions are produced by low loads, a terminal very much stiffer phase, and a transitional region between them in which the skin becomes progressively stiffer with increased extension (Fontanella et al. 2014).

The difference may be due to the area of skin responding to the loads increasing as the load increased, with areas of skin lateral to the area between the loading tabs being progressively recruited to the strained area. It is clear from the results that uniaxial tension cannot be used in vivo to obtain quantitative mechanical parameters, or constitutive relations for the skin. If this limitation is recognised, the test can still be used to provide useful results (Karimi et al. 2015). The directional variation in the mechanical properties of skin was first demonstrated systematically by Langer who showed that oval holes were produced by piercing the skin of cadavers with a circular

cross section (Langer 1978). The directions of the long axes of the ovals produced a regular pattern of lines, which vary slightly between subjects. The results of in vivo uniaxial tension tests shown that the directions of Langer's lines are generally at right angles to the direction in which the skin shows most extension in the compliant phase'. At many sites, surgical incisions made in the direction of Langer's lines gape less, and heal to produce a less conspicuous scar than do incisions made across the lines. The effects of the undefined test boundaries, skin tethering etc. which influence the results of the uniaxial tension test will also influence Langer's lines, which are determined under similar in situ conditions. The in vivo uniaxial tension test provides, therefore, a useful way of determining the specific direction of the Langer's lines in areas of interest to the surgeon.

5.5.3 Mechanical testing of human pelvic tissue

This study focuses on determining the structural and mechanical properties of the pelvic floor connective tissue. The structural composition of this tissue have been previously determined by performing histological analyses (Warren et al. 1990). Tensile tests on POD specimens were conducted and the Young's Moduli of the toe and linear regions of the stress–strain curves were calculated. The mechanical data revealed significant differences between the different patient pathological groups. Due to existing similarities between the histological properties of the porcine and human pelvic floor (Harunari et al. 2006), this investigation provides crucial information on the material behavior of the supportive structures of the male and female pelvic floor in disease.

The axial stress–strain response of the skin and pouch of Douglas displayed the nonlinear strain stiffening phenomenon which is characteristic of soft biological tissues (Rivaux et al. 2013). The tensile behavior of specimens collected from different regions within the pouch of Douglas complex varied greatly. The values (mean+/-Std) of the tangent moduli of the linear region for the POD were found to be 10.2 MPa and 10.4MPa, respectively. The results of the statistical analysis indicated that there was no significant difference in the tangent modulus of the toe or linear region between the POD groups although the control tissue was less stiff. This is likely to be related to the recurrent fibrotic/inflammatory process, which occurs in the tissues over time.

The results of the mechanical tests revealed that the uniaxial stress–strain response of the POD complex within the prolapse groups was similar. The Young's modulus in the linear region and strength of the POD were to be expected given their content of dense connective tissue (Figure 7.0). Because the collagen fibres were oriented mainly along the loading direction in the POD, the mechanical failure was likely determined by the simultaneous rather than progressive failure of the comprising fibres. Such failure mechanisms could explain the lower Young's modulus for the control tissue. The ability of the POD complex to sustain tears and continue to support significant loads is a mechanical feature that warrants further investigation in the future. Different experimental protocols have been used to investigate the elastic properties of human and animal utero-sacral ligament (USL) (Vardy et al. 2005; Rivaux et al. 2013; Martins et al. 2013) found that the mean Young,s modulus and ultimate tensile strength of uterine sacral ligament collected from female cadavers via uniaxial tensile tests were 14.1 MPa and 6.3MPa respectively. Similarly, Rivaux et al.

(2013) performed uniaxial tensile tests on USLs of female cadavers and reported a mean tensile strength of 4MPa (Rivaux et al. 2013). In this study, we identified a mean Young's modulus of 10.8MPa in the linear region. These values are of the same order of magnitude than those reported in the literature (Vardy et al. 2005; Martins et al. 2013). However, major differences in the results may exist due to differences between our experimental methods and previously used ones.

CHAPTER 6

PCR

6.0 Polymerase chain reaction of Fibulin 5, TIMP, MMP1 and MMP9 in patients' skin.

6.1 Introduction

Fibulin-5 is a multi- functional extracellular-matrix protein that regulates cell growth, motility, and adhesion as well as elastogenesis (Schiemann et al. 2002) . Mutations are involved in various disorders such as neuropathies, macular degeneration and in cutis laxa, a disease characterized by loose, redundant skin (Stone et al. 2004) . Moreover, case reports have described prolapse in a subpopulation of women with cutis laxa (Elahi et al. 2006); also mice with null mutation in fibulin-5 (*Fbln5*¡/¡) develop a prolapse that is remarkably similar to that of primates (Wieslander et al. 2009). Tissue processing was performed and PCR techniques as described in 2.8.

Patient Group	Number	Mean age (range) yrs.
Young female control	12	33 (29-35)
Young female prolapse	11	32 (30-32)
Young male control	11	35 (28-43)
Young male prolapse	7	33 (30-36)
Old female control	14	74 (71-82)
Old female prolapse	14	79 (77-82)
Old male control	12	72 (67-82)

Table 6.1. Number of patients whose skin tissue were used for cell culture experiments in relation to gender and age.

6.2 Genes examined

GeNorm algorithm was used to establish the most stable reference genes to which the gene of interest was normalized (see 2.8.2)

6.2.1 Choice of reference genes

Gene	Name	Function	Amplicon base pair
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Carbohydrate metabolism	93
ACT-β	Beta-actin	Cell motility, structure, and integrity	139
RPL13A	60S ribosomal protein L13A	Component of the 60S subunit of ribosome	81
CYC	Cycle	genetic transcription-translation feedback loop that generates circadian rhythms	72
E1FHA2	28S ribosomal protein S2	ribonucleoprotein	81
SDHA	Succinate dehydrogenase, subunit A,	participates in both the citric acid cycle and respiratory chain	82

Table 6.2: Reference genes selected for experiment**.** The sequences used for each gene are given in chapter 2.8.1, 2.8.3

6.2.2 Candidate reference gene expression, stability and pairwise variation

GeNorm statistical algorithms was used for the selection of reference genes for qRT-PCR analysis (Vandesompele et al. 2002). The geNorm is a visual basic application tool, which calculates the expression stability value (M) for all reference genes. The gene with a lower M value suggests its expression is more stable. Additionally, the pairwise variation (Van/Vn+1) of references can also be estimated by using geNorm. It is recommended that no additional genes are required for normalization when the value of Vn/Vn+1 is below 0.15 (Vandesompele et al. 2002). A total of 6 housekeeping genes which have been evaluated in previous studies (Li et al. 2011; Zainuddin et al. 2008) of dermal fibroblasts were chosen as candidate reference genes in the present study.

Based on the Ct values for the 6 candidate reference genes, a boxplot was drawn using GraphPad Prism 5 software to show the expression variation of each gene. The expression stability of the reference genes was analyzed using the geNorm (version 3.52) software packages. To evaluate the gene expression stability, geNorm was used to assess the expression stability (*M*) of each reference gene based on the average pair-wise variation among all of the tested genes (Vandesompele et al. 2002). The lowest *M* value indicates the most stable expression, whereas a higher *M* value is indicative of less stable expression. All of the 23 reference genes had an *M* value below the geNorm threshold of 1.5 and were ranked according to their *M* values in three sample sets. To determine the optimal number of reference genes required for

normalization, pairwise variation analysis (Vn/Vn + 1) was performed using the geNorm software (Figure 6.2). The cut-off threshold value for Vn/n + 1 is 0.15 and no additional reference gene is required for normalization when the value below this value (Vandesompele et al. 2002)In the present study, the value of V2/3 was below 0.15 under all experimental sets (Figure 6.3**)** which suggests that two reference genes would be sufficient for gene normalization in each experimental set.

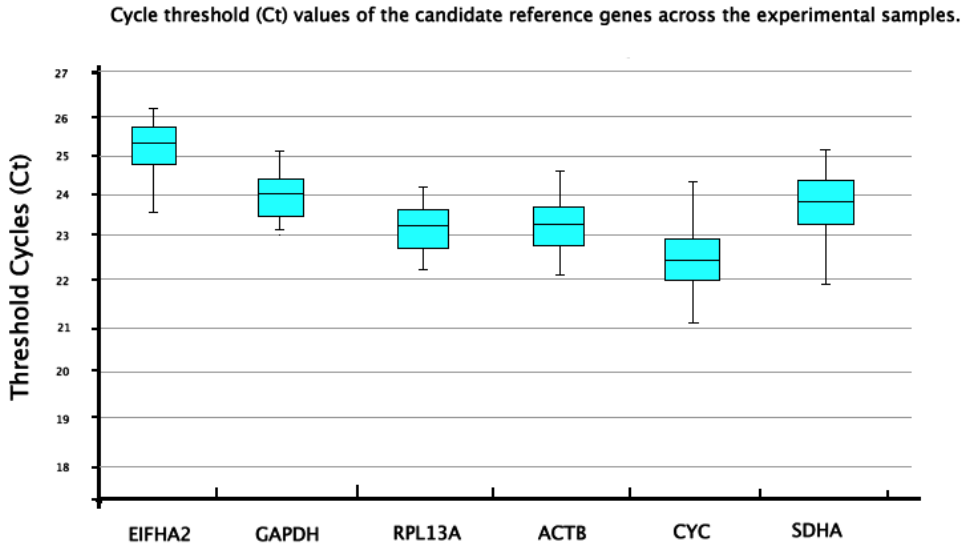


Figure 6.1. Candidate reference gene expression

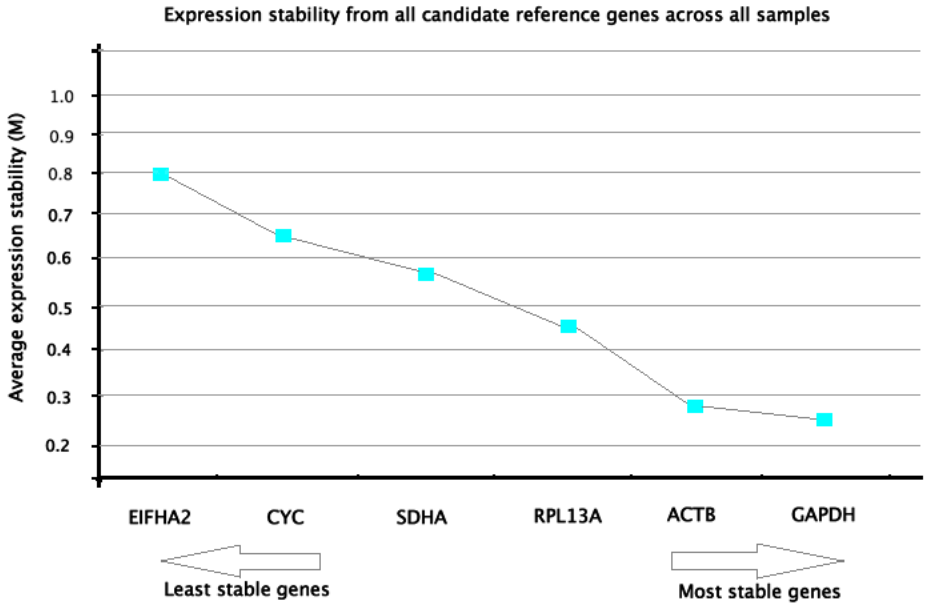


Figure 6.2. Expression stability across reference genes

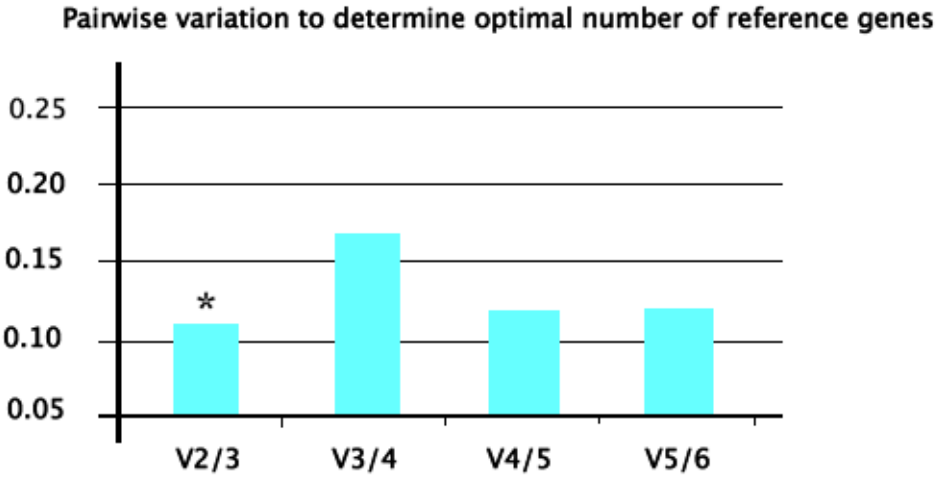


Figure 6.3. Pairwise variation to determine optimal number of reference genes

6.3 Expression of matrix genes in cells cultured from skin samples of patients and controls.

We chose to study fibulin-5 being an integral protein of elastin fibre assembly and MMPs 1,9 and TIMP, which have been previously studied in relation to pelvic organ dysfunction but never in a coloproctology setting. The samples were cultured before gene expression (see chapter 2.9).

6.3.1 Expression of Fibulin-5

FIB-5 mRNA expression was significantly decreased in young men with rectal prolapse compared to young men without prolapse [(FIB-5 mean +/- SD mRNA expression in relative units) 1.26 +/- 0.05 vs. 0.52 +/- 0.20, [P = 0.01]. FIB-5 mRNA expression was lower in young women with prolapse 0.82 +/- 0.07 compared to young women without prolapse 0.96 +/- 0.05 [NS]. FIB-5 mRNA expression was lower in old women with prolapse 0.87 +/- 0.06 and without prolapse 0.98 +/- 0.11 [NS]. Fib-5 mRNA expression was 1.10 +/- 0.04 in older males without prolapse compared with younger males without prolapse 1.26 +/- 0.05 [NS].

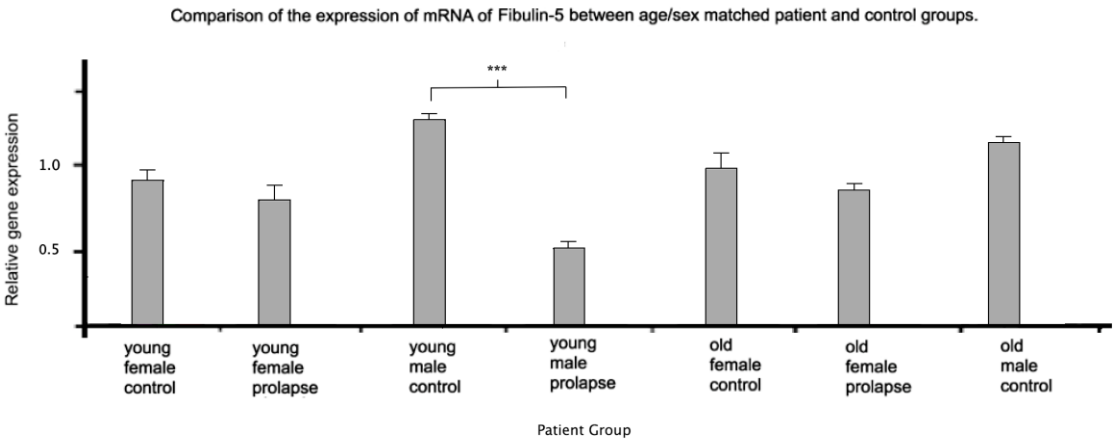


Figure 6.4. Gene expression of Fibulin-5 in relation to age and sex in prolapse patients and in age-and sex-matched controls relative to the reference gene GAPDH.

As the difference of expression was significantly lower in young male prolapse patients than in age- and sex-matched controls, the results for the individual patients in this group are shown below.

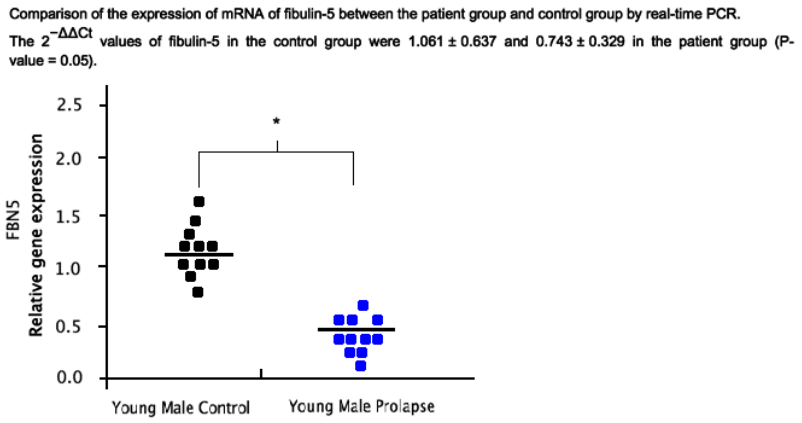


Figure 6.5 Gene expression of Fibulin-5 of individual male patients and controls relative to the reference gene GAPDH.

6.3.2. Expression of TIMP-1 - a metalloproteinase inhibitor

TIMP-1 mRNA expression was increased in young men with rectal prolapse compared to young men without prolapse (TIMP mean +/- SD mRNA expression in relative units) 0.51 +/- 0.09 vs 0.49 +/- 0.02. TIMP mRNA expression was increased in young women with prolapse compared to young women without prolapse 0.26 +/- 0.05 vs. 0.12 +/- 0.04. TIMP mRNA expression was lower in old women with prolapse 0.27 +/- 0.06 and without prolapse 0.28 +/- 0.07. TIMP mRNA expression was 0.31 +/- 0.04 in older males without prolapse compared with younger males without prolapse 0.49 +/- 0.02. The results did not reach statistical significance.

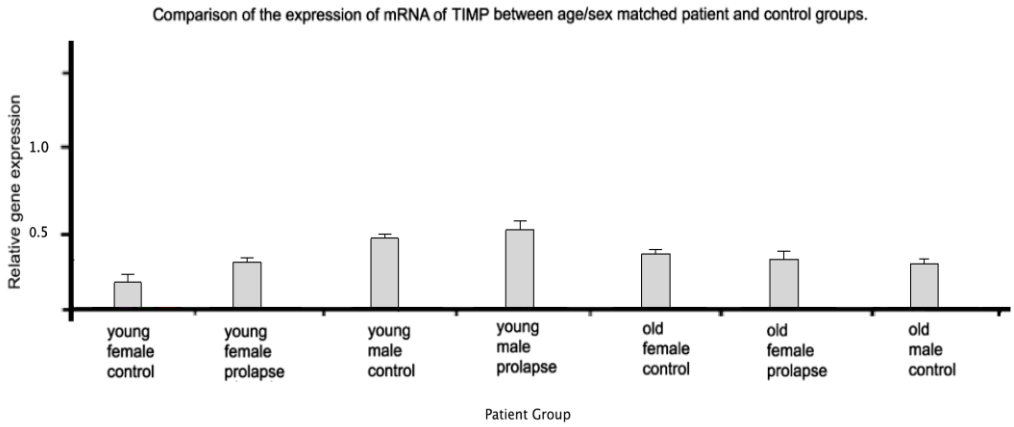


Figure 6.6. Gene expression of TIMP-1 in relation to age and sex in prolapse patients and in age-and sex-matched controls relative to the reference gene GAPDH.

6.3.3. Expression of Matrix Metalloproteinase 1 (MMP1)

MMP1 mRNA expression was increased in young females with rectal prolapse compared to young women without prolapse (MMP1 mean +/- SD mRNA expression in relative units) 0.31 +/- 0.08 vs 0.20 +/- 0.03. MMP1 mRNA expression was decreased in young men with prolapse compared to young men without prolapse 0.42 +/- 0.04 vs 0.59 +/- 0.06. MMP1 mRNA expression was lower in old women with prolapse 0.47 +/- 0.05 and without prolapse 0.52 +/- 0.06. MMP1 mRNA expression was 0.51 +/- 0.04 in older males without prolapse compared with younger males without prolapse 0.59 +/- 0.06. None of the results reached statistical significance.

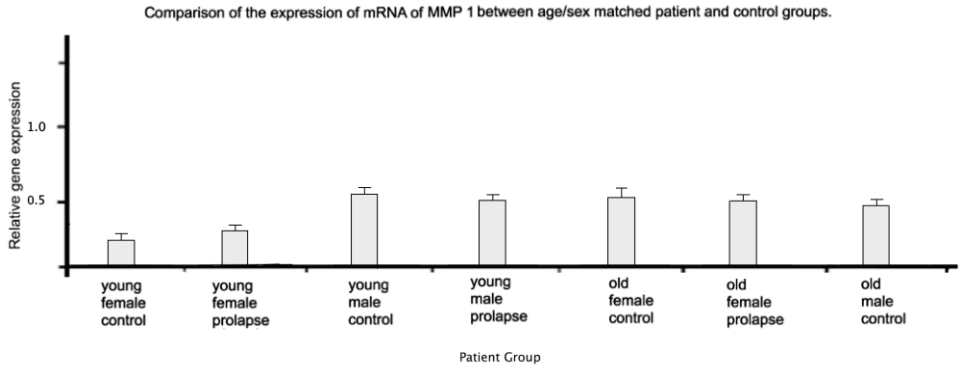


Figure 6.7. Gene expression of MMP-1 in relation to age and sex in prolapse patients and in age-and sex-matched controls relative to the reference gene GAPDH.

6.3.4 Expression of Matrix Metalloproteinase 9 (MMP9)

MMP9 mRNA expression was increased in young men with rectal prolapse compared to young men without prolapse (MMP9 mean +/- SD mRNA expression in relative units) 0.53 +/- 0.08 vs 0.48 +/- 0.03. MMP9 mRNA expression was increased in young women with prolapse compared to young women without prolapse 0.27 +/- 0.05 vs. 0.11 +/- 0.03. MMP9 mRNA expression was lower in old women with prolapse 0.28 +/- 0.07 and without prolapse 0.29 +/- 0.08. MMP9 mRNA expression was 0.32 +/- 0.05 in older males without prolapse compared with younger males without prolapse 0.48 +/- 0.03. The results did not reach statistical significance.

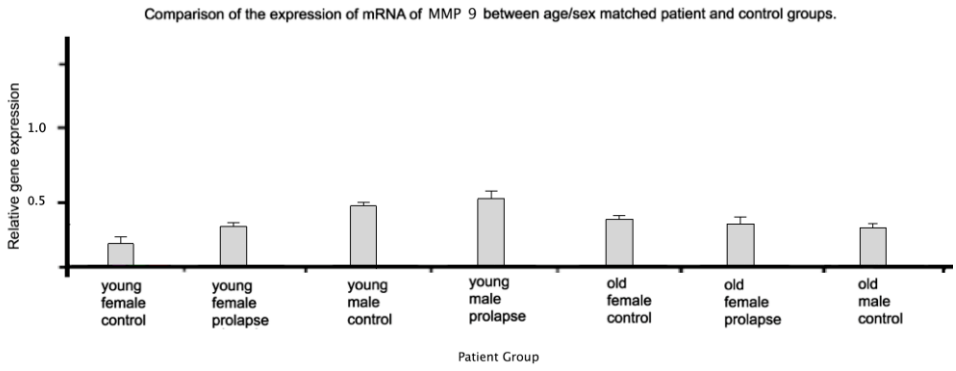


Figure 6.8. Gene expression of MMP9 in relation to age and sex in prolapse patients and in age-and sex-matched controls relative to the reference gene GAPDH.

6.3.5 - Expression of genes of interest in patients relative to controls

We examined the expression of each gene tested in patients relative to controls and shown in Figure 7.8. below which shows that the expression of FBN5 is significantly lower in young males prolapse patients than in young male controls, but that expression of other genes TIMP-1, MMP1 and MMP 9 did not reach statistical significance amongst age/sex matched patients and controls.

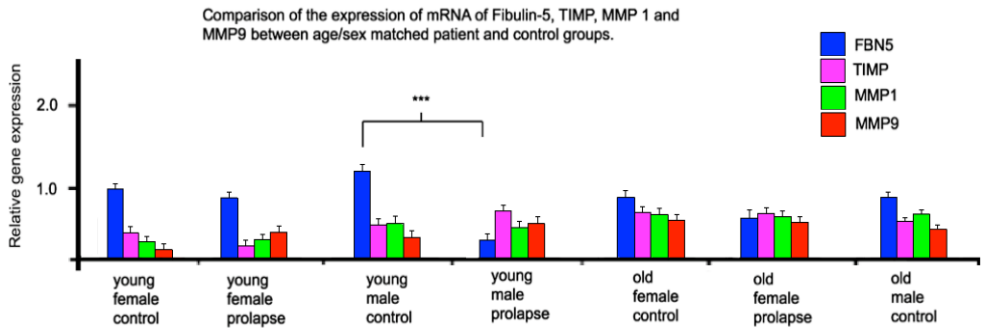


Figure 6.9. Relative expression of genes of interest in patients versus controls

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6.4 Discussion.

The fluorescence-based quantitative real-time PCR (qPCR) has the capacity to detect and measure minute amounts of nucleic acids in a wide range of samples. In 2008, a set of "MIQE" guidelines were proposed to establish a minimum standard for the required information that should be included in publications utilising qPCR experiments (Bustin et al. 2009). In our experiment, we adhered to the MIQE guidelines to the best of our abilities. The RNA extracted from the primary fibroblasts was validated by immunofluorescence, with its quantity, quality and integrity subsequently determined. All RNA meeting the minimum experimental standards was stored at -80°C and was utilised within one month. Primer specificity was confirmed by melting curve analyses. PCR efficiency plots were calculated using a standard curve with the coefficient of correlation (r) between 0.95 and 1.0, and the PCR efficiency of all primers used in this study varied from 0.967 to 1.06. To ensure consistency between reactions, the same quantity of RNA was used, and both RT and qPCR were performed in triplicate. All of these steps ensured the reliability of this experiment and the comparability of the statistical data that was generated.

Another important factor that may affect qPCR results is the selection of the reference genes. *ACTB*, GAPDH are the most commonly used reference genes, but a number of studies have provided solid evidence that their transcription levels vary significantly among different individuals, cell types, developmental stages, and/or experimental conditions (Zainuddin et al. 2008; Wang et al. 2010). Our aim was to identify genes with the lowest expression variability and validate their use for qPCR data

normalisation in expression studies on fibroblasts, employing two different techniques.

The examination of gene expression of proteins involved in the elastic network in these cultures allows us to examine the factors responsible for the normal control of elastin gene expression in this cell system (Avantaggiato et al., 2014), and provides us with important information on the maintenance of the normal elastic fibre network in the skin and other connective tissues. We found that, while there were some indications that expression of MMP9 and TIMP-1was different in controls relative to patients with TIMP-1 and MMP 9 being more highly expressed in young males with prolapse (compared with young male controls) (see figure 7.8) and TIMP-1 being less highly expressed in young females with prolapse (compared with young female controls) (figure 6.6.). Interestingly, MM9 was less highly expressed in younger females with prolapse (compared with young female controls) (see figure 6.8) However the only difference to reach statistical significance was result was that of young male prolapse patients, where expression of fibulin was significantly lower than that of age matched controls (Figure 6.4, Figure 6.5)

It should be noted that the fibulin-5 production in the fibroblast cultures established from an apparently unaffected areas of the skin of the prolapse patients was significantly lower than that in normal, age- and sex-matched control fibroblast cultures. Since the mRNA levels were similar in cell cultures from both patients and controls groups, the relatively reduced fibulin-5 expression in young male prolapse patients was not due to differences in efficiency of RNA extraction. Since cellular

senescence per se appears to reduce elastin production (Sephef, G., and J. M. Davidson, unpublished observations), these higher-passage cultures were eliminated from the study. Lower expression might be due to due to decreased mRNA production or to lower stability of the mRNA post translation, however the mechanism is beyond the scope of this thesis.

The difference in fibulin 5 expressions at the gene level in these young male prolapse patients, is in agreement with the finding of low fibulin-5 immunostaining (Chapter 4.4.3). Thus suggests young male patients with prolapse produce less fibulin-5 protein. It is also notable that the Young's modulus in the skin of young male prolapse patients was lower (Chapter 5.3.2) –. All of these results point to the functional importance of fibulin-5 in the structural stability of skin

CHAPTER 7

DISCUSSION

7.0 Discussion

Study of the skin rather than the pelvic tissues allows us to determine if there are any systemic disorders which maybe precipitating a localised process in the aetiology of prolapse without the confounding effect of the pathology on the tissue of interest (Holbrook & Byers 1989). The results demonstrate an increased density of elastin fibres in patients with rectal prolapse. This difference is also seen with a quantitative difference in the tensile properties of the tissue when stressed (Joshi H et al 2015). The controls correlate with previous work undertaken in the dermatological setting in which dermal elastin fibre composition was found to be around 2.1% (+/- 1.1) (Langton et al. 2010). The study data provides further suggestion of the existence of an occult connective tissue disorder in individuals suffering from rectal prolapse. The extent to which defects in elastic fibre synthesis or function are responsible is unclear at the present time. However, animal models with defects in elastin fibre synthesis are more prone to pelvic organ prolapse (Budatha et al. 2011; Drewes et al. 2007). To date, the limited work that has been undertaken in relation to rectal prolapse, has tended to focus on the potential for a collagenous disorder underpinning the disease (Lammers et al. 2012), whilst this study by no means contradicts those findings, it does suggest that in most cases the aetiology of rectal prolapse is likely to be multifactorial. Indeed, the presence of increased elasticity may exacerbate any existing underlying collagenous predisposition to prolapse. The experimental findings correlate with previous published work describing the presence of elastic fibres in the rectal mucosa of patients suffering from rectal prolapse (Warren et al. 1990).

Observational studies have linked the development of rectal prolapse and disordered

colonic motility to a variety of connective tissue disorders (Jha et al. 2007; Zarate et al. 2010). However, direct analysis of connective tissues is lacking in rectal prolapse research. In related areas such as the development of uterine prolapse, stress urinary incontinence and abdominal hernias, alterations in elastic fibres have been noted (Antoniou et al. 2011). Dermal elastic fibre density did correlate with the presence of external rectal prolapse and this replicates the findings in inguinal hernia development where increased dermal elasticity has been described (Fachinelli et al. 2011). It is possible that increased elasticity in the pelvic connective tissues facilitates the propagation of prolapse due to increased tissue flexibility. Equally it is possible that pelvic fibroblasts synthesise more elastic fibres in response to the prolapse process. The importance of understanding the role elastin fibre abnormalities play in rectal prolapse lies in tailoring treatment to individual patients. Potentially, one might decide on variations on the optimum treatment e.g. whether a mesh should be used at all and if so what is the most suitable material. Furthermore, one might consider making recommendations on mode of delivery to "at risk" females.

In this manuscript we report fibulin-5 expression both at the mRNA and protein levels in the skin of patients with or without rectal prolapse. We find a significant reduction in fibulin-5 expression in skin biopsies from young men with rectal prolapse but no significant change in the female group. Results thus point to an inherent fibulin-5 defect in young men with rectal prolapse.

Human studies regarding fibulin expressions have been studied in pelvic floor connective tissues, exclusively in the urogynacological setting (Takacs et al. 2010; Jung et al. 2009; Söderberg et al. 2009) where findings are inconsistent. Klutke et al

assessed fibulin-5 mRNA expression in the uterosacral ligament of women with uterine prolapse and found fibulin-5 mRNA expression was more than 3-fold greater than in controls but did not assess fibulin-5 expression at the protein level (Klutke J, Ji Q, Campeau J, Starcher B, Felix JC, Stanczyk FZ 2008). Takcas et al however reported a significant decrease in fibulin-5 expression in anterior vaginal wall biopsies of women with cystoceles compared to women without prolapse (Takacs et al. 2010; Kuang et al. 2003) and in the uterosacral ligament of females. The variation samples sources may in part be responsible for the different findings as studies find fibulin-5 gene expression is affected by external conditions. In animal studies, stretch force, when acting on fibroblasts, was found to result in the overexpression of matrix genes including fibulin-5 (Wieslander et al. 2009). The studies to-date thus could show that the up-regulation of fibulin-5 mRNA levels seen by the studies on uterine prolapse are an effect of prolapse rather then its primary cause (De Vega et al. 2009; Papke & Yanagisawa 2014).

The specimens in our study were all prospectively collected using a standardized method with a uniform skin punch biopsy, which could easily be replicated. Most importantly, by contrast, they were taken from an area not affected by the pathology. Thus the changes in fibulin-5 expression seen here are likely to be inherent and thus possibly causal. Animal studies appear to support our hypothesis, that in part, fibulin deficiency or deficiency in the elastin homeostasis may play a role in the development of prolapse [6, 8, 24]. Mice lacking lysyl oxidase-like 1 (LOXL-1), a key protein in the elastic fibre assembly, do not deposit normal elastin fibres postpartum in the uterine tract and subsequently develop pelvic organ prolapse (Klutke J, Ji Q,

Campeau J, Starcher B, Felix JC, Stanczyk FZ 2008). Drewes et al and Budathha et al in studies showed that mice deficient for fibulin-5, important for normal elastin assembly, also developed prolapse(Drewes et al. 2007; Budatha et al. 2011). Serial prolapse examinations in a large colony of *Fbln5*$^{i/i}$ mice showed that 91% of these animals reliably progress to severe POP as a function of age (Schiemann et al. 2002). The observation that Fibulin-5 expression is decreased in young men could explain the significant higher recurrence rate after prolapse surgery in males compared to females in the literature. Recently, Hotouras et al. found even a recurrence rate of 40 % after 6 years following surgery in male patients with rectal prolapse (Hotouras et al. 2014).

7.2 Further work

Clinically, it is seen that women suffering from connective tissue disorders (e.g. Marfan or Ehlers-Danlos syndrome) have higher rates of incontinence and pelvic organ prolapse (Lammers et al. 2012; Carley & Schaffer 2000). Thus, there has been some recent interest in the use of knockout mice to determine the roles of specific matrix proteins in the development of pelvic floor disorders (Liu et al. 2007; X. Liu et al. 2006). Studies by Alperini et al. and others Liu et al 2006, 2007, have recently illustrated that lysyl oxidase-like1 protein (LOX1), an important factor in maintaining elastic fibre homeostasis, is crucial to maintain the structural integrity of the vagina and its supportive tissues (Alperin et al. 2008; X. Liu et al. 2006; Liu et al. 2007). In knockout mice for this gene, the animals would spontaneously develop prolapse following vaginal delivery and with an increase in age. For the 15-month LOXL1 deficient nulliparous animals, 1/3 had severe prolapse precluding biomechanical

analysis, while the remaining animals had mild to no prolapse with the majority of animals exhibiting a prominent perineal bulge. Transanal rectal prolapse was a common finding as well as prolapse of the remaining pelvic organs including bladder, uterus and bowel. Vaginas of the LOXL1$^{-/-}$ mice were circumferentially distended and appeared thinned out, resulting in a genital hiatus (GH) that was larger than wild-type controls, similar to what occurs in women with prolapse. Of the LOXL$^{-/-}$ animals with mild to no prolapse, a 31% decrease in the ultimate load compared to wild-type mice using a uniaxial load to failure test (Liu et al. 2004). Similarly, another recent study has shown inferior biomechanical properties after knocking out the gene encoding fibulin-5 (Fbln5) (Rahn et al. 2009).

Thus, these data collectively suggest that knockouts may be helpful in elucidating the roles of certain proteins in pelvic floor disorders, but it should be noted that these mice are developmentally very different from wild types. Thus, it is difficult to determine primary versus secondary effects. Nevertheless, these animals could play a vital role in helping us determine the mechanism of prolapse in patients with specific types of connective tissue disorders.

7.3 Limitations

However, as noted previously, the challenges we face in obtaining human tissue that are of sufficient size for biomechanical testing makes these comparisons difficult. Not only are surgeons fearful of taking too much tissue, but also they are also fearful of going too deep. Thus, in addition to the plethora of confounding variables that we

cannot easily control in humans, there will always be variability resulting from where the tissue sample was taken, how much was taken, and whether it is full-thickness or not.

7.4 Tissue anisotropy

While determining the mechanical properties of a tissue sample from a uniaxial tensile test can provide meaningful data that allows for comparisons to determine if a specific variable has an impact, it does not always provide an accurate description of how the tissue behaves in vivo if physiologic loads are multiaxial. Tissues have a three-dimensional structure, which is a result of the complex extracellular matrix components that are specifically arranged to meet physiologic demands. Thus, to truly characterize tissue behavior, a test should attempt to simulate in vivo loading conditions as closely as possible. In fact, many tissues are known to be anisotropic, which means that the stress-response will vary depending on the direction the tissue is stretched. This is a common property for biological tissues; for example, skin, blood vessels, heart valves, and vaginal tissue (Metzler et al. 2012). Another common phenomenon is that of axial coupling. In basic terms, this means that the mechanical properties of the tissue along one axis are dependent on the amount of stress or stretch applied to other axes. We attempted to address the question of anisotropy in the skin and pelvic connective tissue to some degree by testing longitudinally and circumferentially cut specimens independently using a standard uniaxial testing protocol.

7.5 Viscoelasticity and viscoplasticity

For most of the mechanical tests described thus far, we have assumed that the tissue behaves as an elastic material. This means that upon loading and unloading of the tissue, the stress–strain curve will follow the same path; or, in other words, if we stretch our specimen repetitively, it will not change its mechanical response over time. In fact, this is not the case for soft tissue. Although these assumptions tend to make analyses easier, it is important to recognize that tissues are in fact viscoelastic/viscoplastic (Cohen et al. 1976; Gennisson et al. 2010). This generally means that the mechanical properties of a tissue are dependent on time and strain history (how it was strained up until and during the experiment). Thus, the stress–strain curves during loading and unloading do not follow the same path. In addition, loading–unloading cycle 1 will be different from loading–unloading cycle 2, and so on. This behavior results from the complex interactions of the collagen, elastin, proteoglycans, and water within the tissue. While accurate cross-sectional area and strain measurements are important for determining reliable stress and strain values, there are also several experimental factors that could influence the stress–strain relationship. Previous, studies have examined the effects of orientation, strain rate, storage, or experimental conditions on many biological tissues (Parente et al. 2009; Tong & Fung 1976; Ottenio et al. 2015; Shergold et al. 2006; Woo et al. 1986; Gleizes et al. 1998). In terms of strain rate, studies in ligaments and tendons have noted that, for mature animals, these tissues are generally insensitive to changes in strain rate (Tong & Fung 1976; Ottenio et al. 2015; Shergold et al. 2006).

Thus, measuring these time-dependent properties can provide us with additional insight into the composition of tissues and allow us to build even more sophisticated models of tissues (Khatyr et al. 2004).

With the utilization of new technologies such as MRI and ultrasound elastography, advanced computational models that incorporate accurate 3-D geometries from dynamic imaging devices, and our improved ability to characterize tissue behavior in the laboratory using biaxial testing, high speed video systems, and lasers to measure tissue geometry, the future of biomechanics research is extremely bright. As our approaches become less and less invasive, direct quantitative measurements of pelvic floor function in humans are becoming more and more of a possibility. Such future advances will likely help us to limit the need for animal models in the future.

7.6 Conclusion

Patients with increased dermal elastic fibres are more likely to develop external rectal prolapse. Our results indicate that these patients may also have an underlying connective tissue disorder contributing to the disease process. The tissue of patients with prolapse has different mechanical properties when compared to the control tissue and this could indicate a disorder of elastic fibre assembly and function.

Fibulin-5 expression is decreased in the skin of young men with rectal prolapse compared to men without prolapse based on our pilot study. Previous animal experiments with *Fbln5*¡/¡ mice and our findings appear to support the hypothesis that decreased expression of fibulins may play a role in the development of prolapse.

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RECTAL PROLAPSE RESEARCH

PATIENT INFORMATION SHEET (version 5)

DATE CREATED: 10/04/2010

RECTAL PROLAPSE AND COLLAGEN ANALYSIS SYMPTOMATIC GROUP

We would like to invite you to take part in a research study. Before you decide whether to participate you need to understand why the research is being done and what it would involve for you. Please take time to read the information sheet carefully and talk to others about the study if you wish to do so.
Part 1 of this leaflet tells you the purpose of this study and what will happen to you if you take part.
Part 2 tells you more detailed information about the conduct of the study.
Ask us if there is anything that is unclear or if you would like more information. Take time to decide whether or not you wish to take part.
You have been invited to participate in this study because you suffer from rectal prolapse and are due to have an operation to treat the condition.

What is the purpose of the study?

Rectal prolapse is a common and distressing condition affecting a large number of adults. It presents either as a protrusion of the rectum (back passage) outside of the body, or as inability to evacuate the bowels normally. The causes of rectal prolapse are not well understood. Three groups of people suffer from rectal prolapse; women with problems related to difficult labour, women who have never given birth to children and men. In women who have had a difficult labour, the muscles that are located inside the

body near the vagina and rectum may become damaged. This can result in prolapse. In men and women who have not had children

this process cannot occur, the reason why these people develop prolapse is unclear and has not been studied.

have I been invited?
You have been invited because you have symptoms of rectal prolapse that will require an operation as part of the treatment.

Do I have to take part?
Participation in research is entirely voluntary. We will explain the study to you and give you a copy of this information sheet. If you are happy to proceed then we will ask you to sign a consent form as a record. If you decide not to take part then your treatment will proceed as normal and will not be altered in any way. You are free to withdraw at any time and do not have to give a reason for doing so.

What will happen to me if I decide to take part?
The normal treatment of rectal prolapse involves an operation. This is usually performed as keyhole surgery. As part of the operation small instruments are placed through the tummy wall and are used to fix the rectal prolapse from the inside. When we do the operation we usually remove the tissue that was attached to and causing the prolapse. Normally this tissue is then thrown away.
If you agree to participate then the operation is performed in exactly the same way. However, instead of throwing the tissue away we want to keep it and study it to see if we can work out why the prolapse has formed. As part of the study we need to get two further samples from you. These are done under the same anaesthetic and at the same time as the operation. One sample is a single 5mm piece of skin that is removed when the keyhole instruments are placed through the tummy wall. This is removed because it will show us what your normal tissues are like. The other sample is a blood test, this will be taken away and we will use it to study the genes that your body uses to tell it how to make the connective tissue that was attached to the prolapse.
These three tests will not cause your operation to be any longer that it otherwise would be. When you wake up from the operation your part in the study will be over and no further tests, meetings or interviews will be required.
The samples will be kept for a period of ten years after the end of the study to allow any additional tests to be undertaken.

What will I have to do?
If you agree to the study then your treatment will proceed in exactly the same way as if you chose not to participate. As all the tests are done at the same time as your operation and under the same anaesthetic.

What are the risks of being involved in the study?
There are no specific risks from being involved in the study. The tissues we are studying would be removed routinely during the operation. The blood test may result in a small bruise, but this is uncommon.

What are the benefits of taking part?
There are no specific benefits for your particular case. However, the information we get from the study will ultimately be used to help other patients suffering from rectal prolapse.

What if there is a problem?
Any complaint that you have about the way the study is conducted will addressed. Detailed information on this is given in part 2.

Will my taking part in the study be kept confidential?
Yes. All the samples are anonymised and only information relating to sex and age is kept.

Part 2
What if relevant new information becomes available?
Sometimes we get new information about the treatment being studied. If this happens, your research doctor will tell you and discuss whether you should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study he may ask you to sign an updated consent form.
If this happens, your research doctor might consider you should withdraw from the study. He/she will explain the reasons and arrange for your care to continue.
If the study is stopped for any other reason, we will tell you and arrange your continuing care.

What will happen if I don’t want to carry on with the study?
You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used.

Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Please contact either Heman Joshi telephone number 07816824343 or Oliver Jones 01865 741841. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or Private Institution). Details can be obtained from the hospital.

What if I am harmed during the study?
In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against John Radcliffe NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised.

Involvement of the General Practitioner/Family doctor (GP)
We will not inform your general practitioner about your involvement in the study as your clinical care will not be affected by your being included in the study.

What will happen to any samples that I give?
For the purposes of this study there are three samples that will be taken. The first sample to be taken is a 5mm piece of skin that will be removed at the same time as we insert one of the keyhole instruments to perform the operation. Although this sample is extra tissue that would not normally be removed at the time of surgery the actual wound is no different in size from the normal keyhole wounds and will be closed in the same way.

The second sample that is taken is the left over tissue that was attached to the prolapse. As previously stated this is usually thrown away and in the study we would keep it for research.
The third sample is a blood test. This would be done at the same time as your operation, while you are asleep. 30ml of blood would be taken and this would be stored for genetic analysis. Some patients notice a small bruise at the site where the blood test was taken, however, this is uncommon
Once taken the samples will be anonymised so that they are not identifiable as being yours. The tissue samples will be tested to determine what types of collagen are present and any chemicals in the tissues that may have caused you to develop the prolapse. The blood tests will be analysed to look at genes which control the way in which your body makes collagen.

Will any genetic tests be done?
Yes they will. The blood samples will have some genetic tests performed on them. These tests cannot be linked back to you or your family and are primarily undertaken to allow us to study how the genetic control of collagen and connective tissue occurs. It will also allow us to see whether some individuals are more prone to prolapse than others.
There would be no implications for your lifestyle or family members arising from these tests. Nor would there be any implications for insurance.
You can refuse to have the genetic tests done and still participate in the main study.

What will happen to the results of the research study?
The results of the study will be presented to other surgeons and scientists. This will allow people to work together to develop new treatments for prolapse and prevent unnecessary repetition of research. No personal details of study participants are retained. This means that we cannot make direct contact with you to let you know the study outcome. However, we would be happy to update you on the progress of the project when you attend follow up appointments.
Who is organising and funding the research?
This research is being organised by Mr. Oliver Jones and Mr. Heman Joshi. money to pay for the research is likely to be donated to us by the Royal College of Surgeons of England and other charities may be approached to provide additional funding.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Reading Research Ethics Committee.

Further information and contact details

For any further information please get in touch with either Mr. Heman Joshi on 07816824343 (e-mail :heman.joshi@nhs.net) or Mr. Oliver Jones on telephone 01865 741841

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RECTAL PROLAPSE RESEARCH

PATIENT INFORMATION SHEET (version 6)
DATE CREATED : 10/4/2010

RECTAL PROLAPSE AND COLLAGEN ANALYSIS CONTROL GROUP

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Part 2 tells you more detailed information about the conduct of the study.
Ask us if there is anything that is unclear or if you would like more information. Take time to decide whether or not you wish to take part.
You have been invited to participate in this study because you suffer from colorectal cancer and are due to have an operation to treat the condition.

What is the purpose of the study?

Rectal prolapse is a common and distressing condition affecting a large number of adults. It presents either as a protrusion of the rectum (back passage) outside of the body, or as inability to evacuate the bowels normally. The causes of rectal prolapse are not well understood. Three groups of people suffer from rectal prolapse; women with problems related to difficult labour, women who have never given birth to children and men. In women who have had a difficult labour, the muscles that are located inside the body near the vagina and rectum may become damaged. This can result in prolapse. In men and women who have not had children

this process cannot occur, the reason why these people develop prolapse is unclear and has not been studied.

Why have I been invited?

During the study we have taken tissue samples from patients with rectal prolapse. However, their tissues are abnormal. In order to work out why people develop rectal prolapse we need to study patients who have normal tissue around their colon.

Do I have to take part?

Participation in research is entirely voluntary. We will explain the study to you and give you a copy of this information sheet. If you are happy to proceed then we will ask you to sign a consent form as a record. If you decide not to take part then your treatment will proceed as normal and will not be altered in any way. You are free to withdraw at any time and do not have to give a reason for doing so.

What will happen to me if I decide to take part?

The normal treatment for colorectal cancer is to remove the cancerous part of the bowel. In addition surrounding normal bowel is also removed. This is to ensure that the cancer is removed completely. Once the bowel has been removed for your tummy we would then take some tissue samples from the specimen that is sent to the pathologist. As part of the study we need to get two further samples from you. These are done under the same anaesthetic and at the same time as the operation. One sample is a single 5mm piece of skin that is removed when we make the skin incision. This is removed because it will allow us to determine whether there are similarities between collagen that is present in the skin is similar to that collagen that is found in the pelvis. The other sample is a blood test, this will be taken away and we will use it to study the genes that your body uses to tell it how to make the connective tissue that was attached to the bowel.

These three tests will not cause your operation to be any longer that it otherwise would be. When you wake up from the operation you part in the study will be over and no further tests, meetings or interviews will be required.

The samples will be kept for a period of ten years after the study is completed in case additional experimental work is needed.

What will I have to do?

If you agree to the study then your treatment will proceed in exactly the same way as if you chose not to participate. As all the tests are done at the same time as your operation and under the same anaesthetic.

What are the risks of being involved in the study?

There are no specific risks from being involved in the study. The tissues we are studying would be removed routinely during the operation. The blood test may result in a small bruise, but this is uncommon.

What are the benefits of taking part?

There are no specific benefits for your particular case. However, the information we get from the study will ultimately be used to help other patients suffering from rectal prolapse.

What if there is a problem?

Any complaint that you have about the way the study is conducted will addressed. Detailed information on this is given in part 2.

Will my taking part in the study be kept confidential?

Yes. All the samples are anonymised and only information relating to sex and age is kept.

Part 2

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, your research doctor will tell you and discuss whether you should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study he may ask you to sign an updated consent form.

If this happens, your research doctor might consider you should withdraw from the study. He/she will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, we will tell you and arrange your continuing care.

What will happen if I don't want to carry on with the study?

You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Please contact either Mr. Heman Joshi telephone number 07816824343 or Oliver Jones 01865 857924. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or Private Institution). Details can be obtained from the hospital.

What if I am harmed during the study?

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against John Radcliffe NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised.

Involvement of the General Practitioner/Family doctor (GP)

We will not inform your general practitioner about your involvement in the study as your clinical care will not be affected by your being included in the study.

What will happen to any samples that I give?

For the purposes of this study there are three samples that will be taken. The first sample to be taken is a 5mm piece of skin that will be removed at the same time as we make the skin incision to perform the operation. Although this sample is extra tissue that would not normally be removed at the time of surgery the actual wound is no different in size from the normal wound and will be closed in the same way.

The second sample that is taken is the left over tissue that was attached to the colon. Usually this is thrown away and in the study we would keep it for research.

The third sample is a blood test. This would be done at the same time as your operation, while you are asleep. 30ml of blood would be taken and this would be stored for genetic analysis. Some

patients notice a small bruise at the site where the blood test was taken, however, this is uncommon
Once taken the samples will be anonymised so that they are not identifiable as being yours. The tissue samples will be tested to determine what types of collagen are present. The blood tests will be analysed to look at genes which control the way in which your body makes collagen.

Will any genetic tests be done?

Yes they will. The blood samples will have some genetic tests performed on them. These tests cannot be linked back to you or your family and are primarily undertaken to allow us to study how the genetic control of collagen and connective tissue occurs. It will also allow us to see whether some individuals are more prone to prolapse than others.
There would be no implications for your lifestyle or family members arising from these tests. Nor would there be any implications for insurance.
You can refuse to have the genetic tests done and still participate in the main study.

What will happen to the results of the research study?

The results of the study will be presented to other surgeons and scientists. This will allow people to work together to develop new treatments for prolapse and prevent unnecessary repetition of research. No personal details of study participants are retained. This means that we cannot make direct contact with you to let you know the study outcome. However, we would be happy to update you on the progress of the project when you attend follow up appointments.

Who is organising and funding the research?

This research is being organised by Mr.Oliver Jones and Mr. Heman Joshi. The money to pay for the research is likely to be donated to us by the Royal College of Surgeons of England and other charities may be approached to provide additional funding.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Reading Research Ethics Committee.

Further information and contact details

For any further information please get in touch with either Mr. Heman Joshi on 07816824343 (e-mail :heman.joshi@nhs.net) or Mr. Oliver Jones telephone 01865 741841.

Oxford Radcliffe Hospitals NHS
NHS Trust

Study Number 5901
Patient Identification Number for this trial

CONSENT FORM (version 4, created 25/04/2012)

Title of Project : **RECTAL PROLAPSE AND COLLAGEN ANALYSIS**

Name of Researcher:

Please confirm by signing your initials in the box provided

1. I confirm that I have read and understand the information sheet dated.....for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2. I understand that my participation is voluntary and that I am free to withdraw at anytime without giving a reason and without my medical care or legal rights being affected.	
3. I agree to tissue samples being taken for research, these will be destroyed ten years after the study is completed.	
4. I agree to blood samples being taken for genetic testing, these will be destroyed ten years after the study is completed.	
5. I understand that relevant sections of my medical notes and data collected during the study, maybe looked at by individuals from regulatory authorities and Oxford Radcliffe NHS Trust. I give permission for these individuals to have access to my records.	
6. I agree to take part in the above study	

Name of Patient	Date	Signature

NHS
National Patient Safety Agency
National Research Ethics Service

ANNUAL PROGRESS REPORT TO MAIN RESEARCH ETHICS COMMITTEE
(For all studies except clinical trials of investigational medicinal products)

To be completed in typescript and submitted to the main REC by the Chief Investigator.
For questions with Yes/No options please indicate answer in bold type.

1. Details of Chief Investigator

Name:	Oliver Jones
Address:	Oxford Radcliffe Hospitals NHS Trust Headington Oxford OX3 9DU
Telephone:	01865741841
E-mail:	Oliverjones10@hotmail.com
Fax:	

2. Details of study

Full title of study:	Collagen Abnormalities and Rectal Prolapse
Name of main REC:	Berkshire- Reading (Previously Milton Keynes)
REC reference number:	**09/H0603/14**
Date of favourable ethical opinion:	24/6/2009
Sponsor:	Oxford Radcliffe NHS Trust

3. Commencement and termination dates

Has the study started?	**Yes**
If yes, what was the actual start date?	1/3/2010
If no, what are the reasons for the study not commencing?	

What is the expected start date?	
Has the study finished? *If yes, complete and submit "Declaration of end of study" form, available at http://www.nres.npsa.nhs.uk/applications/after-ethical-review/endofstudy/*	**No**

If no, what is the expected completion date? *If you expect the study to overrun the planned completion date this should be notified to the main REC for information.*	1/3/2014
If you do not expect the study to be completed, give reason(s)	

4. Site information

Do you plan to increase the total number of sites proposed for the study? If yes, how many sites do you plan to recruit?	**No**

5. Recruitment of participants

In this section, "participants" includes those who will not be approached but whose samples/data will be studied.

Number of participants recruited: 240	*Proposed in original application:500* *Actual number recruited to date:240*
Number of participants completing trial:240	*Actual number completed to date:240*
Number of withdrawals from study to date due to: (a) withdrawal of consent (b) loss to follow-up (c) death (where not the primary outcome) Total study withdrawals:	 0 0 0 0
*Number of treatment failures to date (prior to reaching primary outcome) due to: (a) adverse events (b) lack of efficacy Total treatment failures: * *Applies to studies involving clinical treatment only*	 0 0 0
Have there been any serious difficulties in recruiting participants?	**No**

If Yes, give details:	
Do you plan to increase the planned recruitment of participants into the study? *Any increase in planned recruitment should be notified to the main REC as a substantial amendment for ethical review.*	**No**

6. Safety of participants

Have there been any related and unexpected serious adverse events (SAEs) in this study?	**No**
Have these SAEs been notified to the Committee? *If no, please submit details with this report and give reasons for late notification.*	*Not applicable*
Have any concerns arisen about the safety of participants in this study? *If yes, give details and say how the concerns have been addressed.*	**No**

7. Amendments

Have any substantial amendments been made to the trial during the year?	**No**
If yes, please give the date and amendment number for each substantial amendment made.	

8. Serious breaches of the protocol

Have any serious breaches of the protocol occurred during the year? *If Yes, please enclose a report of any serious breaches not already notified to the REC.*	**No**

9. Other issues

Are there any other developments in the study that you wish to report to the Committee?	**No**
Are there any ethical issues on which further advice is required? *If yes to either, please attach separate statement with details.*	**No**

9. Declaration

Signature of Chief Investigator:	
Print name:	Oliver Jones
Date of submission:	15th June 2012