Chapter 1 Introduction

1.1 Epidemiology of stress urinary incontinence

Stress urinary incontinence (SUI) is extremely bothersome and can lead to significant interference in the quality of life in the female population. It is defined clinically, by the international continence society (ICS), as "the involuntary leakage of urine on effort or exertion, or sneezing or coughing" [1]. This sign may be reproduced by the clinician on examination. In addition to this, urodynamic observations may be undertaken to provide more precise although more invasive evaluations of incontinence. This is typically not required as women may be characterised as suffering from SUI by symptoms and signs alone and conservative measures undertaken to help treat the condition. However, if there is disparity in symptoms or surgical intervention is contemplated urodynamics is currently considered beneficial. The urodynamic definition of incontinence proposed by the ICS is "the involuntary leakage of urine during increased abdominal pressure, in the absence of a detrusor contraction". This is referred to as *urodynamic stress urinary incontinence*. SUI is in contradistinction to urgency urinary incontinence, whereby a sudden compelling desire to void occurs which is difficult to defer. This often shows detrusor overactivity during urodynamic testing. Occasionally both types of incontinence can occur simultaneously.

The definitions above were only coined by the ICS in 2002. Prior to this the definitions of SUI were varied and thus reports of the prevalence of SUI are variable. In a meta-analysis of 48 studies, Hampel *et al*. reported the prevalence of urinary incontinence to be 16% for women younger than 30 years and 29% for women aged 30 to 60 years [2]. The authors found SUI to be more common than urge urinary incontinence, with 78% of women having SUI versus 51% with urge urinary incontinence, with 27% of them having mixed urinary incontinence. In the year 2000, incontinence was responsible for more than 1.1 million office visits in the USA [3]. Also, each year, an estimated 135,000 women undergo surgery for urinary incontinence in the united states alone [4]. The estimated annual cost to the US economy is approximately $19.5 billion [5]. Moreover, it is believed that more than half of women suffering from SUI are embarrassed by the condition, are unable to mention it to their healthcare provider or may even accept some incontinence as part of the ageing process [6].

Incontinence may be characterised by the impact caused by the symptoms on quality of life, by questionnaires like the Incontinence Impact Questionnaire [7]. As a measure of severity, the most widely used indexes are the Sandvik Severity Index [8] and the ICIQ-UI SF index [9]. These have been validated [10].

1.1.1 Aetiology of SUI

The Integral theory of female urinary incontinence, put forward by Petros and Ulmsten, proposes that the cause of SUI is connective tissue laxity in the vagina itself, or in its anterior and/or posterior supporting ligaments [11]. The pelvic floor muscles are unable to compensate for the laxity of the connective tissues sufficiently to maintain closure of the urethra. The levator ani muscles run bilaterally from the superior ramus of the pubis to the spine of the ischium and coccyx. The middle fibres are attached to the rectal sphincter muscles and the anterior fibres descend upon the side of the vagina. In addition to this the arcus tendinius pelvic fascia, termed the "endopelvic fascia" extends from the vaginal apex to perineum and from arcus tendinius to arcus tendinius. Thus pelvic structures are suspended from the endopelvic fascia (Fig 1.1.1.1). Immediately anterior to the proximal urethra are condensations of endopelvic fascia. These are dense enough to be recognised and are termed the pubo-urethral ligaments (Fig 1.1.1.2).

The levator ani muscles, in combination with the endopelvic fascia form an important part of the continence mechanism in women forming a hammock like layer which provides support to the bladder neck (Fig 1.1.1.3) [12]. Contraction of the levator ani pulls the vagina against the posterior surface of the urethra thus closing it [13]. This model is analogous to compression of a hose pipe beneath the foot whilst it is lying on supported and unsupported ground. If the ground is firm good compression may be obtained, whereas a soft ground will lead to inadequate compression. The posterior urethra is intimately connected to the anterior vaginal wall, levator ani muscle complex and the arcus tendineus fascia pelvis. The fascial covering of the levator ani consists of two leaves: the endopelvic fascia (abdominal side) and pubocervical fascia (vaginal side). The two leaves fuse to insert into the arcus tendineus creating a hammock of support suburethrally (Fig 1.1.1.3) [12]. DeLancey also noted that the medial portion of the levator ani had a direct connection with the endopelvic fascia and during increases in intrabdominal pressure contraction of the levators contributed to stabilisation of the urethra.

In a series of magnetic resonance imaging (MRI) studies, it has been shown that the anterior and posterior walls of the bladder neck are pulled apart due to unequal movement of the opposing walls during stress [14] [15]. Anterior support is provided by the pubourethral ligaments working in conjunction with the pubourethralis muscle of the levator ani. Together the two structures form the "midurethral complex", which is thought to also play a significant role in the maintenance of continence. In patients, weakness of the suburethral complex may be sufficient to permit distraction of the anterior and posterior urethral walls during rotational descent.

The other theory previously put forward for the underlying cause of SUI was presented by Enhorning, who proposed that the descent of the urethra outside of the portion of the pelvis affected by intra-abdominal pressure meant no transmission of intra-abdominal pressure onto the urethra. This has however been disputed [12]. Women with SUI commonly show radiological features suggestive of impairment of urethral support such as an increased urethrovesical angle [16], bladder neck hypermobility, reduced functional urethral length [17] or disruption of periurethral ligaments or defects in endopelvic fascia [18]. In summary, the essence of urethral support in women is multifaceted and both endopelvic fascia and pelvic floor muscle tone acting under neural control are responsible for continence.

In addition to urethral supports two arches of striated muscle (compressor urethrae and urethrovaginal sphincter) run over the distal urethra, causing the urethral pressure rise usually seen preceding a rise in abdominal pressure [13]. In women, integrity of the intrinsic urethral sphincteric mechanism is a major factor in maintenance of continence. The urethral sphincter is composed of intrinsic urethral smooth muscle and extrinsic striated muscle components and it extends throughout the proximal two-thirds of the urethra. In certain patients an intrinsic sphincteric deficiency exists leading to incomplete sphincter closure thus predisposing to SUI [19] [20]. Blaivas *et al*. first classified primary urethral weakness into three types [21]. Although the classification still remains in the literature it has been largely superseded by the term "intrinsic sphincter deficiency", focussing attention on urethral elements which include pudendal innervation, striated sphincter mass and function, urethral smooth muscle, mucosa and submucosal cushions [22]. It is believed that both intrinsic sphincter weakness and para-urethral tissue weakness leading to urethral hypermobility may co-exist to some degree in women with SUI [23]. Urodynamic studies, in particular urethral pressure profilometry, may be utilised to diagnose pure intrinsic sphincter deficiency. However, currently these procedures do not comprise routine practice [24].

1.1.2 Risk factors for SUI

Consequently risk factors, leading to weakness of either endopelvic fascia or pelvic floor musculature, have been investigated in an effort to increase understanding of the condition. It is evident that ageing is associated with a higher risk of SUI [25]. SUI increases up to the age of 50, thereafter it is followed by little increase in the levels of prevalence [26]. The mechanism in these cases may be the effect of the loss of muscle tone, long term effects of denervation injuries sustained during childbirth or changes in hormonal status leading to alterations in collagen in paravaginal tissues [27] [28]. However, it has proved difficult to show a link between natural menopause and increasing risk of SUI [29] [30]. Also the evidence for hormone replacement therapy does not show it to reduce the risk of the development of SUI as evidenced by two RCTs [31] [32] [33].

Likewise, obesity has been cited as a risk factor, with women who have a higher body mass index displaying higher rates of SUI [34]. Women in the highest quartile of body mass index are two to four times more likely to have urinary incontinence than those in the lowest quartile [31]. This is postulated to be consequent upon the existence of a greater abdominal pressure and/ or laxity in support tissues caused by chronic strain. Smokers have been shown to have between 1.8 and 2.9 times the relative risk of SUI as a non-smoker [34] [35]. Alarmingly, the

above three risk factors are all on the increase. This will further propel the prevalence of this condition in the future adding to the pressing requirement for prevention and adequate cure.

The other major risk factors are pregnancy and vaginal delivery [36], which have been found to lead to SUI postulated to be due to denervation of the pelvic floor sphincter muscles, which progresses during a period of many years [37] [38]. The data concerning the actual risk are conflicting with some studies indicating no statistically increased risk with pregnancy [39] or vaginal delivery [40]. However, some studies have demonstrated that women who gave birth via caesarean section were at increased risk of SUI compared to nulliparous women and women who gave birth vaginally were at an even greater risk than those giving birth via the caesarean route [25] [41]. Epidemiological studies have shown approximately half of all women develop transient urinary incontinence during pregnancy and post partum the prevalence of urinary incontinence is still 9-31% [22]. Moreover, 40% of primigravid women have a history of occasional SUI before becoming pregnant. With this being the case, it is noted, that the SUI invariably worsens during pregnancy itself rather than post parturition [42].

Medical co-morbidities, such as, the length of time with type II diabetes mellitus have been found to confer increased risk of SUI [43]. Other co-morbidities presenting risk include neurological disorders and connective tissue disorders [44] [45]. Hysterectomy was feared to impose a significant risk, however most studies have failed to show an association with SUI [46] [47].

1.2 Epidemiology of pelvic organ prolapse

Similar to SUI, pelvic organ prolapse (POP) is an extremely troublesome condition. Studies vary as to the exact prevalence of POP, which is reported to be between 3.6% -11.4% [48] [49]. Each year, an estimated 225 00 women undergo surgery for POP in the united states [50]. The lifetime risk of undergoing a surgical procedure for SUI or POP by age 80 was reported at 11.1%, 30% of these requiring additional surgical procedures for recurrence of the same condition [51] [52]. More recently, in an Australian study, the lifetime risk for a woman of requiring POP surgery was reported at 19% [53]. Pelvic floor disorders affect nearly one third of pre-menopausal women and nearly half of post-menopausal women [51].

In 2001, POP was defined by the National Institute of Health as “the leading edge of any vaginal segment being $\geq$ 1cm above the hymenal remnants [54]. The following year the ICS

defined POP as the descent of one or more vaginal segments; anterior, posterior and apex or vault after hysterectomy [55]. This led to the POP quantification system (POPQ):

Stage 0: no prolapse

Stage 1: more than 1 cm above the hymen

Stage 2: within 1 cm proximal or distal to the plane of the hymen

Stage 3: more than 1 cm below the plane of the hymen but protrudes no further than 2 cm less than the total length of the vagina

Stage 4: complete eversion of the vagina

Thus we see two august bodies with different definitions of normality and abnormality. Some studies have reported between 0.2-11% of asymptomatic patients having vaginal segments at or beyond the hymenal remnants, with 35% having POPQ stage 2 [56] [57] [58] [59] [60]. The other known grading system used for cystocele is the Baden-Walker halfway system:

I: bladder descent toward introitus with strain

II: bladder to introitus with strain

III: bladder outside introitus with strain

IV: bladder outside introitus at rest

Although we have begun to classify and describe genital tract prolapse in a recognised and comparable fashion, this still has no real bearing on what symptoms the patient may have and what is considered normal [61] [62]. A study by Gutman *et al*. found vaginal descent 0.5cm beyond the hymen predicted bulging symptoms but no correlation was found with other symptoms [63]. A combination method of symptomotology and quantifiable grading is still sought.

There are three compartments for prolapse:

Anterior compartment prolapse- is a prolapse of the bladder, urethra or both into the vagina, resulting in a cystocoele, urethrocoele or cystourethrocoele respectively (Fig 1.2.1).

Middle compartment prolapse- is a prolapse of the uterus or herniation of the pouch of Douglas (containing omentum/small intestine- enterocoele) into the vagina. In addition to this, women post hysterectomy can develop a vault prolapse (Fig 1.2.2).

Posterior compartment prolapse- is a prolapse of the rectum into the vagina (Fig 1.2.3).

1.2.1 Aetiology of POP

DeLancey has provided a good description of the connective tissue support of the vagina, dividing the support into 3 levels (Table 1.2.1.1) [64] . Level 1 provides support to the upper 1/3rd of the vagina and is comprised of the uterosacral and cardinal ligaments (Fig 1.2.1.1). Level 2 provides support to the midvagina, consisting of the arcus tendinius fasciae, which attach laterally extending to the pelvis. Level 3 describes the support to the distal vagina by the perineal body and perineal membrane. In addition to the pelvic ligaments; the paravaginal tissue, termed endopelvic fascia, is one of the mechanisms responsible for pelvic floor strength. Underlying the vaginal serosa it extends from vaginal apex to perineum and from arcus tendinius to arcus tendinius. A tear or weakness in this layer leads to herniation of the underlying tissues into the vagina.

Structure	Failure / Defects	Anatomical result
Uterosacral ligaments (Level 1 support)	?Disruption, ?overdistension and elongation	Uterine prolapse Posthysterectomy vault prolapse
• Anterior endopelvic fascia • Lateral attachment at arcus tendineus fascia pelvis with proximal attachment at ischial spine (Level 2 support)	• Attenuation of fascia Disruption from attachment	• Midline cystocele • Paravaginal defect-cystocele • Uterine or vault prolapse
Perineum (Level 3 support)	Disruption from endopelvic fascia Disruption of bulbocavernosus muscles	Excessive perineal descent Rectocele
Levator ani muscle • Reduced tone/attenuation • Perineal descent • Vertical course of vagina	• Disruption/avulsion from pubic ramus • Paravaginal defect-cystocele	

Table 1.2.1.1 Structural elements of pelvic organ support, possible damage and resulting failure (reproduced with permission from Delancey [64]).

Histologically, the pelvic floor ligaments are composed of connective tissue containing; collagen, elastic fibres, proteoglycans, extracellular matrix (ECM) and smooth muscle [65]. The vaginal wall is composed of 4 layers; the squamous epithelium, the thin subepithelium- composed of dense collagen, the muscularis- a fibromuscular layer providing longitudinal support, the adventitia- loose connective tissue surrounding the vagina. The muscularis layer is the layer that is plicated during prolapse repair. The tensile strength in these tissues is determined by the amount of collagen I in respect to collagen III and IV [66]. An increase in collagen I leads to an increase in tensile strength of the tissue, conversely excess collagen III and IV decrease mechanical integrity [67]. Menopause seems to decrease the collagen I/III ratio [68] [69].

Paravaginal tissues contain approximately 84% collagen and 13% elastin. Elastin provides the tissues with passive recoil after stretch. Altered elastin metabolism and gene expression have been found in women with POP [70]. Patients with pelvic floor dysfunction also have been found to have structurally altered tissue, particularly in reference to collagen [71] [72] [73] [74]. Tenascin, a glycoprotein involved in tissue repair has also been found in uterosacral and cardinal ligaments of prolapsed uteri [75]. This finding points towards remodelling secondary to biomechanical stress as predisposing to POP. In addition, women with POP have also been found to have less organised and smaller bundles of smooth muscle, with loss of smooth muscle content correlating with degree of prolapse [76] [77]. A more in depth discussion of the histological changes is presented in section 1.11.

Nichols and Randall described two types of anterior vaginal wall prolapse: distension and displacement [78]. Distension is thought to result from overstretching and attenuation of the anterior vaginal wall, caused by overdistention of the vagina associated with vaginal delivery or atrophic changes associated with aging and menopause. The distinguishing physical feature of this type was described as diminished or absent rugal folds. The other type, displacement, was attributed to pathologic detachment or elongation of the anterolateral vaginal supports to the arcus tendineus fasciae pelvis, resulting in descent of the anterior segment with the rugae intact.

1.2.2 Risk factors for POP

Intact innervation of the levator ani muscle, anal and urethral sphincters is required for normal pelvic function. Electromyogrpahic studies performed before and after childbirth induced pelvic floor denervation showed increased fibre density after parturition [79] [80]. Ageing leads to further deterioration of pelvic floor denervation. Prolapse stage has been found to be increased by pregnancy and shown to persist in women post vaginal delivery [81]. Mant *et al*. analysed a database of over 17, 000 women attending the Oxford family planning clinic and reported that women with a history of 2 or more pregnancies had a relative risk of requiring surgery to correct POP of 8.4 compared to nulliparous women [56]. Vaginal birth has long been associated with POP [82] [83]. However, delivery via caesarean section has only been shown to be partially protective[40]. In multivariate analyses, a birth weight greater than 4kg has been shown to increase the risk of POP [83] [84].

In a population based cross sectional study looking at non obstetric risk factors; BMI, family history, heavy lifting at work (>10kg) and constipation were all significantly and positively

associated with the development of POP [85]. Another study found family history to confer a risk of 1.4 times normal [86]. Comparative studies have reported a 30% familial incidence of POP [87]. Genetic polymorphisms, such as the laminin-C1 (LAMC1), conferring risk of POP have been documented [88]. In mice it has been identified that the gene responsible for the formation of the uterosacral ligaments was the Homeobox-A11 (HOXA11). In women with prolapse, HOXA11 and collagen expression were shown to be significantly decreased [89].

Epidemiological studies have demonstrated age as a major risk factor for POP [90] [91]. However, the interaction of age, menopause and hormonal status seems inseparable. Weight loss was not found to significantly cause regression of POP in a prospective study, suggesting damage to the pelvic floor related to excess weight may be irreversible [92]. Smoking has not been found to confer a significant risk [93]. Women with connective tissue disorders such as Ehlers Danlos and Marfan's syndrome, as well as women with neurological disorders are predisposed to developing POP [45] [94].

1.3 Treatment of SUI

1.3.1 Non surgical treatments

The National Institute of Clinical Excellence (NICE) recommends lifestyle changes such as weight loss and supervised pelvic floor exercises as first line therapy for the treatment of SUI [95]. Failing this, electrical stimulation and biofeedback may be used. However, in one study, electrical stimulation has not shown to be effective compared to sham treatments in patients with the ability to contract pelvic floor musculature [96]. Conversely, Castro *et al*. reported benefit in patients receiving treatment via pelvic floor exercises, electrical stimulation or vaginal cones [97]. The subjective improvements were of the range of 54-58% for all three treatment modalities compared to 21% in an untreated group. However, the urodynamic parameters stayed the same which may suggest a placebo effect. Pelvic floor exercises have been shown to improve symptoms of SUI and have also been recommended for prevention of POP in nulliparous women prior to pregnancy [61]. Moderate weight loss has been shown to decrease SUI symptoms [98].

Women with SUI have lower resting urethral pressures than age matched continent women [99] [100]. Therefore, pharmacological methods to increase urethral pressure seem logical. Urethral tone is maintained by the release of noradrenaline on to the alpha adreno-receptors [101]. Many different alpha adreno-receptor agonists have been used to treat SUI. The most widely used were ephedrine and norephedrine [22]. Alhasso *et al*. reviewed the evidence for

alpha adreno-receptor agonists and found limited evidence to recommend their routine use [102]. The evidence suggested that drugs were better than placebo at reducing pad usage and improving subjective parameters. Beta adreno-receptor antagonists have been postulated to enhance alpha adrenoreceptor action by beta adrenoreceptor blockade. However, the clinical benefit of this has not been sufficiently reported [103].

Duloxetine, a serotonin nonadrenaline reuptake inhibitor has been licensed for the treatment of women with SUI. It is understood to work on the urethral rhabdosphincter [104]. Both noradrenaline and serotonin are thought to lead to enhanced contraction of the sphincter via a potentiating effect on glutamate [105]. Women taking Duloxetine for eight weeks were found to have a significantly higher mean urethral pressure profile, maximal urethral closure pressure but not functional urethral length [105]. A double blind, randomised, placebo controlled study in 533 women found Duloxetine to significantly decrease incontinence episode frequency in 64% of women at a dose of 80mg/day [106]. At that dose 15% of women discontinued the drug due to non severe side effects most commonly nausea. Further clinical trials have also led to this conclusion [107-109]. Higher doses have been associated with psychiatric disorders which has limited its popularity.

A Cochrane review concluded subjective cure to be 10.8% with duloxetine compared to 7.7% for placebo [110]. Moreover, surgery is still considered more cost effective than Duloxetine [95]. It is postulated Duloxetine will help correct sphincter disturbances, however, urethral mobility due to poor suburethral tissues will remain uncorrected and therefore women with a dominance of this leading to their SUI will have less benefit from Duloxetine. Imipramine works in a similar fashion to Duloxetine. A 35% cure rate and 50% improvement have been reported in patients with this [111]. However, there exist no RCTs on the effects of Imipramine.

Oestrogens play a significant role in the continence mechanism. In addition to oestrogen receptors existing in the vagina, levatores muscles, ligaments and fascia, oestrogen plays an important role in the maintenance of a positive urethral pressure [22]. Exogenous oestrogens have been shown to reduce collagen concentration and decrease collagen cross linking in urogenital tissues [112]. A recent Cochrane review has not shown sufficient benefit from local oestrogen therapy and systemic therapy has been linked with worsening of SUI [113].

As lack of collagen is thought to be a cause for pelvic floor disorders, many researchers have attempted to inject collagen para-urethrally, to act as a urethral bulking agent. A Cochrane review found peri-urethral collagen injections for SUI to have a reasonable short term cure [114], but a multicentre randomised trial found a 19% reduced success rate with collagen

injection compared to surgery [115]. Isom-Batz and Zimmern discuss the use of collagen injections in patients after a failed suspension procedure. Outcomes were good with 93% claiming improvement or cure with just over half of patients only requiring one injection [116]. However, one must bear in mind that this is a complex population of patients and cost effectiveness analysis has found collagen injection to be beneficial only as second line treatment after failed surgery [117]. Other urethral bulking agents have been used ranging from autologous fat, carbon particles, calcium hydroxyapetite, ethylene vinyl calcohol copolymer, dextranomer and silicone based Macroplastique®. The latter has been found to have the greatest efficacy over the others [118].

Macroplastique® is a urethral bulking agent involving injection of cross-linked polydimethylsiloxane. It is a soft, flexible implant, which upon implantation is encapsulated in fibrin. The carrier gel is absorbed leaving the fibrous capsule, which is thought will not migrate [119]. A systematic review found success rates of 46% and 88% [120]. Recently Ghoniem *et al*. reported a multicentre single blinded RCT comparing collagen injection to Macroplastique® [121]. The cure rate at 1 year was 36.9% of patients in the Macroplastique® group compared to 24.8% in the collagen group. The risk of urinary retention was 3.2 and 6.6% and urethral erosion was 0.8 and 1.6 % respectively. Given the cure rates are less than that reported for surgery, many surgeons would not use bulking agents as first line. However, it may be considered for use as second line after failed surgery. Moreover, if used as a first line treatment, many note an adverse effect on consequent surgical intervention.

1.3.2 Surgical treatments for SUI

Surgery is often the treatment modality of choice for SUI. There are 5 main categories of repair:

1. Anterior vaginal repair/ anterior colporrhaphy with bladder buttress (Fig 1.3.2.1). Anterior vaginal repair is used to treat prolapse, if associated with incontinence then buttress sutures are placed on the bladder. This plication provides support to and/or elevates the urethra and/or bladder neck. Many variations of the procedure exist. Meta-analysis have reported the success rate of this procedure to be in the region of 67.8-72% [122]. The serious complication rate is 1% and the incidence of detrusor overactivity 6%. At 5 years the continence rate was found to decline to just 37%, thus making this procedure unpopular [123] [124].

2. Burch Colposuspension is a longstanding effective treatment. The top of the vagina is lifted and fixed with permanent stitches into the space behind the pubic bone (Fig 1.3.2.2). It is reported as having a continence rate of 83-87% at one year [125] [124]. A 51% objective cure rate has been reported at two years by Ward and Hilton in a multicentre randomised trial [126]. At five years the authors reported a 90% negative pad-test amongst women in the colposuspension arm of the trial [127]. Complications reported from this study showed a rate of 39.8% of POP after this procedure and reoperation rates for incontinence of 3.4%. The procedure may also be undertaken laparoscopically but currently is not recommended for primary SUI [128].

3. Needle suspension procedures use suspending sutures and patch materials to suspend the bladder neck, usually from bone anchors placed in the pubic bone. These are seldom used due to poor success rates [129]. At a mean follow up of 5 years of 42 patients Tebyani et al. found a subjective success rate of 5%, improvement in 12% and failure in 83%.

4. Tape procedures use synthetic materials as a tape placed beneath the mid urethra without tension. The tape procedure has enjoyed much success in recent years due to the minimal invasiveness of the technique. There are three main methods of positioning the tape, depending on its length. A full-length tape passes through the retropubic space, underneath the urethra to the other side, and is fixed by sutures to the anterior abdominal wall. Shorter tapes are attached by suspending sutures at each end of the tape to the anterior abdominal wall. Alternatively, bone screws may be used to secure the sutures into the pubic bones. The tape can also be brought round through to the obturator internus muscle and out of the obturator foramen, termed the transobturator tape (TOT). Once the sling is in position, a cystoscopy may be performed to check that there has been no bladder perforation. The simplified variations of the procedure are shown in Fig 1.3.2.3.

   Over the years many different companies have begun to market their tapes, which are made of different materials. By far the most common prosthesis used is the tension free vaginal tape (TVT- Ethicon®). More than 1.4 million procedures have been performed worldwide with this. There are many different kits and operative variations available marketed from the numerous companies selling these tapes. The transobturator tapes are either placed from inside to out (TVTO) or outside to in (TOT) Fig 1.3.2.4.

The objective success rates of the TVT are 63% at two years and improvement is noted in another 20% [130]. In the updated meta-analysis by Novara et al. the objective cure rates for the TVT were higher than the TOT but the subjective cure rate was the same and TOT led to a decreased risk of intraoperative complications [131]. The success rates are similar to that of the pubovaginal sling but the latter was found to lead to a higher rate of storage lower urinary tract symptoms (LUTS).

5. Pubovaginal slings have been used for many years. Price described the first rectus fascia sling in 1933. The sling is placed beneath the bladder neck and the amount of tension on the sling requires an individualized approach. A sling which is too tight may lead to urinary retention and conversely a sling that is too loose may lead to recurrence. Tissue may be obtained from many different sources such as autografts, allografts and xenografts. The characteristics of the various materials used in all of the above are considered later in the thesis.

   The success rates of pubovaginal slings vary according to the type of material used. The success reported with cadaveric fascia is mixed, ranging from 33 to 93% [132] [133] [134]. The rectus fascia sling has a success rate of approximately 80% depending on the level of patient selection and follow up [135] [136]. The success rates with porcine dermis have been found to be 54% at 36 months follow up [137]. However, Abdel-Fattah *et al*. found no difference in improvements in cure, defined by improvements in quality of life, between porcine dermis and TVT [138].

1.3.2.1 Complications of SUI surgery

Complications of SUI surgery may be divided into intraoperative and postoperative. Intraoperative complications involve injury to organs, vessels and nerves. The injury may occur to:

1) Urethra- This is a rarely reported complication. It may occur during transvaginal dissection or trocar placement. Failure to recognise this leads to the risk of urethrovaginal fistula development and sling erosion/infection.
2) Bladder- Bladder injury varies with the surgical approach. Albo *et al*. reported more injuries with colposuspension compared to sling procedures [139]. At meta-analysis the risk of bladder perforation is less with TOT compared to TVT (p=0.0007).

3) Ureter- ureteric injury is very uncommon, but the ureter may be kinked during a Burch colposuspension. The position of the midurethral sling makes it extremely unlikely for the ureters to be encountered.
4) Blood vessels- multiple blood vessels traverse the deep pelvis, these are at risk in the obturator fossa and pelvic side walls. The Iliac vessels and vascular pedicle of the bladder are at risk especially during blind passage of trocars or needles.
5) Bowel- bowel injury is very rare, only being reported in case reports [140]. It may occur during retropubic dissection for a burch colposuspension, during the retropubic dissection for a pubovaginal sling or during trocar passage for a midurethral sling.
6) Nerve injury- may occur during harvest of rectus fascia or during trocar movement in the pelvis [141] [142] .

Postoperative complications include:

1) Voiding dysfunction and urinary retention- symptoms range from difficulty voiding to complete retention and/ or urgency incontinence. Post-op voiding difficulties lasting more than four weeks occur in 4-8% of burch colposuspension procedures and 3-11% of sling procedures and permanent retention was estimated to occur in less than 5% of patients [143]. Surgical intervention for voiding dysfunction has been reported in 0-5% of women after midurethral sling procedures [140] [144].
2) Vaginal extrusion and urinary tract erosion- extrusion implies the presence of sling in the vaginal cavity and erosion refers to the appearance of sling in the urinary tract. Extrusion rates have been found to be higher with multifilament slings as opposed to monofilament [145] [146]. The risk of extrusion with monofilament slings is less than 5% [147]. The risk of erosion is reported to be less than 1% [144] [147] [148] [149].
3) Infection- multiple case reports of pelvic abscesses have been reported post SUI surgery [150] [151] [152] [153] [154].

1.3.2.2 Outcome measures of SUI surgery

Comparable scientific assessment is essential for the evaluation of SUI surgery, to allow comparison between procedures and guide best practice. The commonly used methods of both objective and subjective assessment are discussed below. Any outcome measure must be reliable, valid, interpretable and responsive to change.

Objective measures:

1) Urodynamics- has been found not to show SUI in 15% of women with primary SUI [155]. Post operatively, Azam *et al*. demonstrated almost 40% of women, who complained of persisting SUI, did not show leakage on urodynamics [156]. Although urodynamics is the gold standard for the objective assessment of SUI, its reliability is not proven.
2) Cough stress test- may be used to assess cure, but may not be used to assess improvement [157]. Prior to commencing the test, bladder volume, number and force of coughs should be standardized. However, currently no standards exist.
3) Pad tests- have been used to objectively define cure, using a cut off depending on the test duration and standardization. The 1 hour, 24 hours and 72 hours pad test have been validated [158] [159] [160].
4) Bladder diaries- have been shown to be a reliable measure but rely on the duration of the test and are currently not standardized [161] [162].

Subjective measures:

1) ICIQ questionnaire- is a modular questionnaire which has been validated, shown to be reliable and is used in many research studies [128]. The ICIQ modular questionnaire is an amalgamation of other existing questionnaires. The modules range from core symptom modules, quality of life modules and optional modules.
2) Satisfaction outcomes- ascertain from patients their satisfaction from a certain intervention. This relies heavily on patient goals and expectations and Norton has shown a reduction of leaks by 50% does not entail a 50% improvement in satisfaction [163].

1.4 Treatment of POP

Current treatment options for POP involve pelvic floor muscle training, use of pessaries (mechanical devices such as rings or shelves), and surgery.

1.4.1 Non surgical treatments

Pelvic floor muscle training requires skilled support. Hagen *et al*. randomised 47 women with POP to either supervised physiotherapy or a lifestyle advice sheet. There were both objective

(45%) and subjective (63%) improvements in symptoms, which were better than the control group [164]. A Cochrane review concluded the evidence for pelvic floor muscle training is still limited and requires further robust study [165]. Moreover, it has been found, less than one quarter of women with POP can perform adequate muscle contractions at the time of initial evaluation [166].

A survey of gynaecologists in the United States found pessaries were most frequently used in patients deemed to be poor surgical candidates [167]. Also vaginal pessaries were found to be commonly used with topical oestrogens. These are occasionally prescribed pre-operatively to prevent the progression of pelvic organ prolapse [168]. In a study by Fernando *et al*. from 203 women who chose a pessary for treatment of their POP, 15% discontinued treatment within 2 weeks. Urinary symptoms were improved in approximately 30% of women, but there were no improvements in SUI symptoms [169].

1.4.2 Surgical treatments

The surgical treatments for the different types of POP are considered separately as three groups.

1.4.2.1 Upper vaginal prolapse (Uterine & vault)

Uterine prolapse occurs due to weakening of the uterine support ligaments. As this occurs most commonly in women above the age of 50, hysterectomy with vaginal apex fixation has been the treatment of choice. For younger women, wishing to preserve their fertility a sacrohysteropexy may be performed, where the uterus is attached to the sacral bone in a tension free fashion providing support for the uterus. In the U.S.A the most common indication for hysterectomy in the over 55 age group was found to be uterine and vaginal prolapse [170]. When comparing vaginal hysterectomy with abdominal sacrohysteropexy, Roovers *et al*. found significantly more complications in the abdominal group [171].

Vaginal vault prolapse is a late complication of hysterectomy and has an incidence of 0.2-4.3% [172]. For the treatment of vaginal vault prolapse, Richter first described the vaginal sacrospinous colpopexy for suspension of the vaginal vault [173]. This procedure involves suture suspension, either unilaterally or bilaterally, of the vagina from the sacrospinous ligament resting the upper vagina over the levator plate. The main concern with this procedure

is the fact that the vagina is placed in an exaggerated horizontal position, which increases force on the anterior compartment with increases in abdominal pressure. This is increasingly likely if a concomitant anti-incontinence procedure is performed. This non physiologic axis likely results in a higher rate of cystocele formation, reported at around 20% to 30% [174].

The sacralcolpopexy involves vaginal fixation through suspension material (mesh) to the anterior longitudinal ligament of the sacrum (Fig 1.4.2.1.1). Lane later reported the sacralcolpopexy as an abdominal approach. The abdominal sacralcolpopexy uses a mesh to suspend the vaginal vault retroperitonealy. Maher and colleagues [124] in their meta-analysis combined 3 studies [175] [176] [177] which showed abdominal sacralcolpopexy was better than vaginal colpopexy with a better surgical outcome and less recurrence of vaginal prolapse or occurrence of SUI postoperatively. The abdominal operation was however longer and led to a slower return to activities of daily living [175].

For use in the sacralcolpopexy, Culligan *et al*. compared the use of absorbable cadaveric fascia lata with non-absorbable polypropylene mesh [178]. There were no recurrences of vault prolapse but 14 out of 44 women in the fascial group and four out of 45 in the mesh group developed prolapse at another site. Also two out of 54 women in the mesh group developed mesh erosion. A recent review noted the rates of erosion to be 2-11% for sacralcolpopexy using synthetic mesh [179]. Allograft fascia has been found to have no risk of mesh erosion but results in high failure rates, often with no evidence of mesh remaining at reoperation [132] [180].

1.4.2.2 Anterior vaginal wall prolapse (cystocele, urethrocele, paravaginal defect)

Many forms of anterior vaginal repair exist. Typically, the anterior vaginal repair (anterior colporrhaphy) involves an incision in the anterior vaginal wall, reduction of the bulge followed by plication of the paravaginal fascia (Fig 1.4.2.2.1). Thereafter, a mesh/graft may be placed over the repair to induce fibrosis and strengthen the repair (Fig 1.4.2.2.2). The graft may also be used to suspend the vaginal wall from the pelvic side walls. Finally the vaginal wall is closed over the graft. Techniques for mesh/graft application include: overlays, modified four-corner attachments, transobturator attachments, and anterior flaps as part of an apical mesh procedure. A Paravaginal repair may also be used to treat anterior wall prolapse. This involves attachment of the lateral vagina to the arcus tendinius fascia either abdominally or vaginally. Success rates vary from 75-97% [181] [182].

At meta-analysis Jia *et al*. looked at 10 randomised controlled trials involving 1148 women [183]. There was evidence that the use of any mesh/graft was better than no mesh/graft at preventing objectively determined recurrence of anterior prolapse, 14% Vs 30% respectively. When Non-RCT data was included, procedures not using mesh had the highest failure rate (29%), followed by absorbable synthetic (polyglactin) mesh (23%), porcine dermis graft (18%), and non-absorbable synthetic polypropylene mesh (9%). This trend appeared to be supported by the need for re-operation (for recurrent and new prolapse), which was highest in women treated with absorbable synthetic mesh (9%) compared with 3% for biological grafts and 1% for non-absorbable synthetic mesh. The median follow up of the above studies was 13 months. Jia *et al*. also concluded that the data comparing different types of grafts/ mesh for posterior vaginal prolapse repair were too few to perform meta-analysis.

Another meta-analysis by Foon *et al*. reported a lower risk of objective recurrence after one year in the patients having an anterior repair with a biological adjuvant material (odds ratio 0.56) and absorbable synthetic adjuvant material (odds ratio 0.44) [184]. The erosion rates amongst studies using non-absorbable and absorbable synthetic adjuvant material were 14% (21/150) and 2.9% (1/35) respectively, whilst amongst the studies with biological adjuvant material it was 0.67% (1/150). The number needed to treat with biological adjuvant material to prevent one recurrence at 12 months post-operatively was 13 and with absorbable synthetic adjuvant material it was six.

Mesh contracture had been reported by Gauruden-Burmester in 192 women who had anterior or posterior mesh placement [185]. Introital ultrasound measurements revealed polypropylene (PPL) mesh contracted on average from 7.5cm to 3.5cm (54%) in the anterior compartment compared to and 11.5cm to 6.4cm (46%) in the posterior compartment. The authors found that this was not associated with altered post-op vaginal length measurements.

1.4.2.3 Posterior vaginal wall repair (rectocele)

There are three common approaches to repairing a rectocele. A posterior colporraphy involves a midline plication of the vaginal bulge thus tightening the posterior vaginal fascia. A levatorplasty involves plication of the levatores muscles over the rectum. A site specific repair involves specific plication of the paravaginal defect and in some cases addition of a graft. A vaginal or anal approach may be undertaken to perform the repair.

At meta-analyses, the results for posterior vaginal wall repair were better than trans-anal repair in terms of subjective (RR 0.36) and objective (RR 0.24) failure rates. Analysing women with rectocele alone showed that recurrent rectocele occurred in 2/39 in the vaginal group and 7/48 following the trans-anal repair, a difference that did not reach statistical significance. Postoperative enterocele was, however, significantly less common following the vaginal surgery as compared to the trans-anal group [124] .

Sand *et al*. compared posterior repair with and without Vicryl absorbable mesh [186]. Rectocele recurrence (graded according to modified Baden-Walker) appeared equally common with and without mesh augmentation. However, no subjective outcomes were measured. Transperineal rectocele repair using polyglycolic acid led to an 89% anatomical cure rate at 14 months follow up as well as improvements in functional outcomes [187]. Synthetic grafts used for the traditional posterior colporraphy are associated with higher anatomical success rates but carry the risk of higher complication rates [188] [189].

1.4.2.4 Complications of POP surgery

Complications of POP surgery may be divided into intra and post-operative. Intra-operative complications are similar to those of SUI discussed earlier. Damage may occur to local structures depending on the approach and procedure.

Post-operatively there is a risk of occult or de novo stress incontinence [190]. Also there is a risk of urgency incontinence, although it is believed prolapse repair improves this risk [191] [192]. In addition, there is a risk of gastrointestinal complications such as ileus and small bowel obstruction post abdominal sacrocolpopexy [193].

1.4.2.5 Outcome measures of POP surgery

Scientific evaluation post surgery for POP is essential to allow comparisons between studies and guide clinical practice. Methods of assessment are divided into objective and subjective.

Objective measures:

1) Clinical examination and measurement using the POPQ or Baden walker halfway grading systems. Unfortunately these anatomical measurements may not bear a significant relationship to patient symptoms and although a patient may have a degree of persistence of prolapse after a procedure they may be symptom free [194]. In addition to this un-operated areas of the vagina may seem unsupported but again be asymptomatic. Moreover, attempting for POPQ-stage 0 (perfect vaginal support) is an unrealistic aim and is inconsistent with the demographic profile of asymptomatic parous women [195].

Subjective measures:

1) There are three main questionnaires that have been validated to assess POP. These are the Pelvic Floor Distress Inventory (PFDI), Pelvic Floor Impact Questionnaire (PFIQ) and the electronic Personal Assessment Questionnaire (ePAC) [128]. The questionnaires assess a range of symptoms and quality of life parameters.

1.5 Simultaneous SUI and POP

Although women with anterior vaginal wall prolapse may also experience SUI, women with advanced prolapse may not have apparent SUI symptoms. This is due to the phenomenon of urethral compression or kinking leading to an increase in maximum urethral closure pressures [196]. Conversely 40% of women with urethral sphincter incompetence will have a significant cystocele. Occasionally referred to as “occult” or “latent” incontinence, the appearance of SUI during the valsalva manoeuvre after reduction of the prolapse in the absence of a detrusor contraction, is the urodynamic definition of the condition. Therefore occult SUI will also present after correction of the prolapse and its incidence is thought to range between 36-80% [197]. The risk of de novo SUI following POP repair (patients with a negative preoperative reduction cough stress test) was shown to be 1.9% [190].

The long standing question of performing prophylactic continence procedures during POP surgery or embarking upon POP surgery whilst inserting mid-urethral slings has been looked at

on the Netherlands TVT database [198]. TVT and POP surgery was performed in 59 women compared with 687 women having TVT alone. Of the 59 all had SUI but not all had POP. The tape was equally successful in all patients whether a prolapse procedure was required or not. Another study followed 1356 women undergoing sling procedures for SUI and found concomitant procedures were performed in 34.4% [199]. Women undergoing a dual procedure were less likely to require a repeat procedure for their SUI in the first year post sling insertion. Also these women were less likely to undergo surgery for POP in the first year; however, the occurrence of outlet obstruction was significantly higher (9.4% Vs 5.5%) in this group.

A randomised trial, which was commenced to evaluate the benefit of dual prolapse and incontinence surgery in patients with prolapse who develop occult incontinence on manual reduction of the prolapse prior to surgery, was terminated early (after the first 232 women had been randomised, but data were finally available for a total of 322 women) because of a significant difference in the incontinence rates at three months after surgery. The addition of Burch colposuspension to abdominal sacrocolpopexy significantly decreased the incidence of SUI three months after surgery (44% Vs 24% with Burch). However, the operating time was longer and the blood loss higher in the Burch group [186].

Abou-Elela *et al*. looked at the use of the transobturator tension-free vaginal mesh and TVT-O procedures for patients with SUI & anterior vaginal prolapse [200], 18/20 patients had optimal anatomic results and two patients had stage 1 prolapse at a mean of eight months. All patients were simultaneously cured of urinary incontinence and were satisfied on questionnaire. Besides one patient with perivesical haematoma, treated conservatively, no other complications were noted. Meschia *et al*. demonstrated that adding a TVT led to better two year objective continence results compared to adding endopelvic plication of the urethrovesical junction to a cystocoele repair [201]. Complications were not different.

1.6 Prostheses in SUI/ POP

The purpose of the sub-urethral sling is to provide support to the posterior urethral wall. The rationale for mesh use in POP surgery comes from repair of abdominal wall hernias. Hernia recurrence rates are much lower if mesh is used to repair it. Meta-analysis of 10 randomised controlled trials has shown that the use of any mesh to treat POP led to a decrease in recurrence rates[183]. It follows that the patient's tissues are already weak and to stitch these

together as is done for a colporrhaphy, without providing extra support or the potential for fibrosis leads to increased risk of recurrence. An ideal adjuvant material is one that has:

1. Good tissue integration- non-carcinogenic, resistant to infection and non-allergenic i.e. biocompatible.
2. Good biomechanical characteristics similar to native healthy tissue.
3. Good surgical characteristics i.e. easy to handle and suture, and cost effective.

The reaction of soft tissues to implanted biomaterials is variable. Four types of response have been identified [202]:

- A minimal response, with a thin layer of fibrosis around the implant.
- A chemical response, with a severe and chronic inflammatory reaction around the implant.
- A physical response, with an inflammatory reaction to certain materials and the presence of giant cells.
- Necrotic tissue: a layer of necrotic debris is produced, resulting from in situ exothermic polymerization.

The first type of response is what we generally aim for. Histologically four stages are seen over time [203]:

- Stage 1- In the first week, an intense inflammatory infiltrate around the implant, capillary proliferation, granular tissue and the presence of giant cells containing birefringent material.
- Stage 2- After two weeks the granular tissue remains and histiocytes appear, with more or fewer giant cells.
- Stage 3- After four weeks, the acute inflammation disappears, capillaries reduce in number, and the number of histiocytes and giant cells increases.
- Stage 4- Some giant cells are present on the external surface of the implant with dense, fibrous tissue present.

Adjuvant materials can be classified by material type, weave and pore size and may be biological or synthetic. Biological adjuvant materials (grafts) come in the form of allografts, autografts and xenografts. Autografts are taken from the patient themselves (e.g. fascia lata from the thigh). Allografts are taken from the same species while xenografts are taken from another species (e.g. porcine dermis/ small intestine submucosa). Synthetic adjuvant materials may be classified as absorbable and non-absorbable and the pore size classified as macro-porous (>75µm) and micro-porous (<75µm). Type 1, large pore synthetic monofilament has

been recommended for use in SUI/ POP [204] [205]. It is thought that knitted monofilament, lighter weight, larger pore size, increased porosity, and decreased density are associated with improved host tolerability and, consequently, lower rates of exposure, erosion, and infection. We describe the different prostheses available and their current problems. Thereafter we will describe our proposed tissue engineered solution to these problems.

1.6.1 Absorbable synthetic prostheses

The most commonly used are Dexon (polyglycolic acid) and Vicryl (polyglactic acid). These implants are absorbed by the body and are proposed to stimulate fibroblast activity to stimulate long lasting fibrosis. Absorption of mesh occurs via macrophages digesting the mesh material, this is replaced by healthy scar tissue with newly formed collagen fibres [206]. Vicryl takes 30 days and Dexon 90 days to be absorbed. Unfortunately clinically, absorbable meshes have performed less well for treating prolapse compared to non-absorbable meshes [186] [207]. However, a recent meta-analysis reported absorbable synthetic material to have a lower objective recurrence rate at 12 months compared to no mesh but this data is only short term [184]. The use of absorbable sub-urethral slings is minimal and with limited follow up [208]. Absorbable meshes are associated with less erosion or infection.

1.6.2 Non Absorbable synthetic prostheses

Many varieties of these exist and are classified according to structure, material composition and pore size, classified into four types by amid below [209]:

- Type I: Completely macroporous mesh (Atrium, Marlex, Prolene and Trelex). The pore size exceeds 75 µm, the size required for infiltration by macrophages, fibroblasts, blood vessels in angiogenesis and collagen fibres.
- Type II: Totally microporous mesh (Gore-Tex, surgical membranes). The pore size is smaller than 10 µm in at least one dimension.
- Type III: Macroporous patch, with multifilaments or a microporous component: Polytetrafluoroethylene (PTFE Teflon), woven Dacron (Mersilene), woven polypropylene (Surgipro), perforated PTFE (Mycro Mesh).
- Type IV: Biomaterials with submicron pores (Silastic, Cellgard, dura mater substitute). These materials are often associated with those of type I to prevent adhesion in intraperitoneal implantation.

When pore sizes are smaller than 10µm, bacteria measuring 1µm may be admitted into the mesh and be protected from macrophage attentions due to the larger size of these cells which cannot enter the mesh. Thus type II and III meshes are vulnerable to infection and type I less so as they admit macrophages, especially fibroblasts and allow for angiogenesis. Thus, theoretically infections should be treatable in Type I meshes without the need to remove the implant. Moreover, histological analysis has revealed a decreased level of inflammation and fibrosis when large pore mesh is used. Macroporous mesh with a pore size of greater than 100µm is thought to allow fibrous tissue through the mesh thus allowing adhesion formation, while biomaterials that contain a pore size of less than 75µm may be more prone to being encapsulated rather than infiltrated by the host tissue [210] [211] [212].

The structure may be; woven, leading to good strength and memory but poor conformity and fraying of material, or knitted, giving flexibility and conformity but compromising strength. In addition to this, surfaces may be a composite of materials where one material may provide strength and the other flexibility. Thereafter materials may be multifilament or monofilament, wrapped or braided.

At meta-analysis non-absorbable synthetic meshes have a lower objective prolapse recurrence rate than absorbable meshes- 8.8% Vs 23.1% respectively, but the rate of erosion was 10.2% compared to 0.7% respectively [213]. When polypropylene (PPL) tape was explanted from 10 women requiring revision of their original surgery, no mesh degradation had occurred [214]. PPL had the greatest degree of host tissue infiltration with neovascularisation. However a foreign body reaction was evident by the presence of giant cells, macrophages and occasional calcification. TVT is the most common sling used for incontinence repair, with reported rates of success of 85% and a patient satisfaction rate of 70% at 7 year follow up [215]. Erosion rates vary according to the type of synthetic material used and the type of procedure performed, ranging from 2% to 10.7% [216] [217] [218] [219], with synthetic materials having an erosion rate 15 times greater than allografts [220]. The risk of erosion was found to be significantly less with the type I PPL monofilament sling as compared to the type III multifilamentous PPL tape [216]. This may be due to better integration of the type I macroporous tape, allowing better integration into host tissue.

1.6.3 Autologous prostheses

Autologous prostheses are obtained from the patient themselves. These are preferred in younger women due to there being less perceived risk of erosion in the longer term. They are,

however, associated with morbidity to the harvest site [221]. Autologous tissue causes no immunogenic reaction and over time collagen remodelling has been demonstrated in it [222]. This is supported by histological analysis by Woodruff *et al*. which showed moderate degradation of the grafts and also showed moderate and uniform infiltration of host fibroblasts, as well as neovascularisation. There was no evidence of inflammatory cell infiltrate or foreign body reaction. Autologous material displayed no evidence of encapsulation (fibrous rim of tissue surrounding and isolating the graft) [214]. Although initial success rates in incontinence repair are good, in the longer term failure rates increase and at five years Guerrero *et al*. report only 50% success rates [223]. When autologous tissue has been used to augment enterocele repair, at 22 months follow up, 90% of patients were subjectively satisfied with their outcome and objective rates of persistent enterocele were 1.7%, cystocele 15% and vault prolapse 8.3% [224]. Adjusting for different follow up periods Howden *et al*. found the recurrence of incontinence post autologous sling repair to be five recurrences per 100 women-years [225].

1.6.4 Allograft prostheses

These are usually of cadaveric origin and can consist of fascia lata, dermis or dura matter. They are freeze-dried and gamma irradiated to render them non-antigenic thus tissue typing is not required prior to implantation. Upon re-exploration of the failure of these, many authors describe allografts as being absent [226] [222]. Woodruff *et al*. showed high levels of degradation and moderate levels of encapsulation [214]. The peripheries of the grafts were invaded by fibroblasts but the central portions remained acellular. This fits with the clinical picture as medium term (four to 12 months) results of cadaveric fascia allografts have shown unexpectedly high rates of failure [227] [228]. Adjusting for different follow up periods Howden *et al*. found the recurrence of incontinence post cadaveric graft repair to be 16 recurrences per 100 women-years [225]. Similar results were also found by McBride *et al*. at two months follow up where 42% of patients receiving a cadaveric allograft had urodynamic stress incontinence although the subjective improvement rate was higher [229]. When comparing cadaveric fascia lata to synthetic mesh the optimal surgical outcome was only achieved in 61% of women in the former group compared to 89% in the latter suggesting its inferiority [230]. Erosion rates vary between 0% [180] [231] to 11% [232]. In addition to this cadaveric tissues have the added risk of virus or prion transmission albeit small.

1.6.5 Xenograft prostheses

These come from other species and as allografts are gamma sterilised and may be crosslinked. The majority are porcine in origin, either porcine dermis (PD), or small intestinal submucosa (SIS). PD grafts have been found to be severely encapsulated at explantation from women, being entirely separate from periurethral tissue. These grafts were completely acellular without evidence of host infiltration or neovascularisation [214]. This is demonstrated clinically in sub-urethral sling procedures by the high failure rate. Giri *et al*. reporting a failure rate of 46% at 36 months follow up [137]. In addition to this, Lucas *et al*. began to compare TVT Vs Pelvicol® (porcine dermal collagen). The authors had to stop the Pelvicol® arm of the RCT due to high failure rates at both 6 and 12 months [233]. When looking at patients who underwent cystocele repair and augmentation with PD graft, 13% had recurrence of cystocele at 24 months follow up [234]. Recently in a randomised study, Feldner *et al*. found significantly higher anatomical cure rates (86% Vs 59%) by using a SIS graft compared to traditional colporrhaphy for anterior prolapse at 12 months follow up [235]. However there were no differences in the quality of life of the two groups.

1.6.6 Tissue engineered prostheses

Attempts at the manufacture of living tissue engineered prostheses have only reached an early stage. We could not find any reports of these reaching patients for clinical evaluation. However, in vitro, Skala *et al*. have described the methodology of vaginal fibroblast isolation and attachment to different meshes [236]. Starting with $1cm^2$ of vaginal tissue, which was subsequently cut into smaller pieces and incubated with collagenase A in buffer overnight they went on to centrifuge at 300g for 7 minutes. The cell sediment was re-suspended in DMEM and into T25 flasks. Each mesh was then seeded with 10 million cells per $2\text{-}3cm^2$ at passage three to five. The cells were positively stained for vimentin and negatively stained for actin, and cytokeratins five, six, and 14, which is in keeping with fibroblasts. Propidium iodide staining also helped quantify the extent of fibroblast attachment at five hours and five weeks post seeding. Seven meshes were assessed for fibroblast attachment and the biological xenograft meshes (Sugisis and Pelvicol) had the most fibroblast attachment at five hours and five weeks. This was followed by Obtape (thermo-annealed PPL). Surgisis showed some degeneration in structure over five weeks.

Similarly, Langer *et al*. attached human fibroblasts on to three PPL based meshes [237]. They found light weight, thin filament meshes were better for fibroblasts to attach to. Also

fibroblasts attached to mesh nodes and around thin filaments. Polyglactin (absorbable Vicryl) allowed for better cell attachment. Interestingly, microporous meshes had a better cell coating than macroporous meshes.

Kapishke *et al*. attached 300 000 foreskin fibroblasts to three different PPL meshes [238]. They found fibroblasts settled on PPL threads and developed long extensions with a ruffled surface. SEM also revealed fibroblasts were capable of deposition of extracellular matrix containing type I and III collagen, with type I collagen forming an extended network of collagen fibres. Contineza *et al*. found human dermal fibroblasts concentrated around PPL thread intersections, whereas in polyester meshes they surrounded the multifilamentous threads [239].

Another research group, Cannon *et al*, have made tissue engineered slings from muscle derived stem cells on an SIS scaffold [240]. Histological staining showed these cells could migrate and form differentiated myotube structures with spontaneous contractile activity at four and eight weeks. The authors also used a rat model to assess sling properties by assessing the effect on leak point pressure in rats with and without slings. The tissue engineered slings were found to have no adverse effect on leak point pressure.

Mitterberger *et al*. injected autologous myoblasts and fibroblasts in 123 women with SUI [241]. The cells were obtained from skeletal muscle biopsies, suspended in a collagen carrier material. Fibroblasts were injected into the urethral submucosa and myoblasts into the rhabdosphincter. Patients with mild urethral hypermobility were excluded. After a follow up of one year 79% were subjectively cured and 13% improved. All patients received injections on average of 3.8×10^7 fibroblasts and 2.8×10^7 myoblasts. The same group also used the same treatment in 63 men with stress incontinence post prostatectomy and showed improvement in 17 and a return of continence in 41 [242]. These results appear excellent but it must be noted the same group had a randomised controlled trial publication, comparing injection of collagen with myoblasts and fibroblasts, retracted from the Lancet for failure to comply with ethical guidelines. The investigators questioning this study had concerns whether the published RCT actually existed [243].

From the above studies we see the recognition of the need to attach fibroblasts to meshes to aid tissue integration. We see much work still needs to be done to develop a methodology to easily isolate fibroblasts, attach them to meshes and quantify the number of cells required for this purpose. Moreover, research is needed to define the effect this has on the mesh and ultimately the effect of this *in vivo* and on patient outcome.

1.6.7 Design of a tissue engineered prosthesis (TEP)

Given the success and complication rates of current prostheses, described above, it is clear much work is still required to optimise the prostheses used in SUI/ POP surgery. The idea of a TEP has recently attracted some attention and will continue to do so as our understanding of both prostheses and tissue engineering science increase. I have described the sought after characteristics of a prosthesis above and with these as our aim we ask the question "what are the key and necessary requirements for the design of a tissue engineered prosthesis?" Fig 1.6.7.1 shows the design inputs and the output characteristics of the ideal TEP. Essentially, we need to identify the scaffold, cells and culture conditions for a TEP.

1.7 The scaffold

This thesis will attempt to increase our understanding of a good scaffold material. Much work is required in this regard. The scaffolds upon which cells have attached well, in the literature to date, are porcine dermis (PD), porcine subintestinal submucosa (SIS) and polypropylene (PPL) (Section 1.6.6). Therefore we will include these three scaffolds and another four; cadaveric dermis (CD) and Alloderm (AL)- these have been used for SUI/POP surgery as acellular biological scaffolds and, thermoannealed Poly(L)-Lactic acid (Th PLA) which is a favourable scaffold used extensively in tissue engineering, and sheep forestomach (SF)- as it has been proposed to have good biomechanical properties. These scaffolds will be assessed for the outputs shown in Fig 1.6.7.1.

1.8 The cell

The question of which cell is beneficial for the creation of a tissue engineered prosthesis is an important one. Thus far in the literature, dermal fibroblasts, vaginal fibroblasts, foreskin fibroblasts and muscle derived stem cells have been used (section 1.6.6). Given the majority cell type in the endopelvic fascia (the connective tissue amongst which prostheses are placed) are fibroblasts, it seems intuitive to use fibroblasts. However, the source of the fibroblasts is under much debate. One might assume that autologous fibroblasts from the host site would be ideal. However, there is concern in the field regarding the use of fibroblasts from affected tissue. In fibroblasts from the anterior vaginal wall of women with SUI, Chen *et al*. showed a pro-metalloproteinase state and these fibroblasts could not be stimulated to produce tissue

inhibitors of metalloproteinase by oestrodiol [244]. In contrast, fibroblasts from women without prolapse showed higher levels of tissue inhibitors of metalloproteinase and were stimulated by oestrodiol. Also in other studies, myofibroblasts (differentiated from fibroblasts) from women without POP led to greater contraction of collagen gels, than myofibroblasts from women with POP [245].

We were unable to find any studies of the investigation of oral fibroblasts in women with SUI/POP. However, encouragingly, skin from women with SUI/POP has been shown to be normal and not easily extensible [246]. This implies that the skin fibroblasts that produce this skin are also normal and their use is therefore probably not precluded. In addition, autologous cells clearly remove the risk of an adverse immune reaction.

Stem cells are gaining popularity in the field of tissue engineering. Broadly speaking, two types of stem cells have been identified; adult and embryonic. The latter as the name suggests are derived from an embyo and have the ability to differentiate in to all cell types i.e. are totipotent. Despite their promise, preliminary trials with these have led to problems such as tumour formation [247]. Adult stem cells, also known as tissue derived stem cells, have lesser differentiating potential but can still be stimulated to differentiate into mature functioning cells. This is independent of their source up to a degree [248] [249]. Adipose derived stem cells, due to their abundance and ease of extraction are also becoming a popular choice [250]. However, In contrast to fibroblasts, mesenchymal stem cells require guidance to differentiate and this can take the form of culture chemicals [251] [252], structured scaffolds [253], adjunctive cell culture [250] or manipulation of the physical environment [254].

In this thesis, we will only use buccal mucosa fibroblasts. This is because we have extensive experience with their use in the laboratory along with clinical experience in human subjects [255]. They are also easy to access and lead to minimal morbidity which could be beneficial when taking this work forward to the clinic. In addition, the literature base on the differentiation of stem cells producing connective tissue is still only being developed and hence there are, as yet, no studies of long term clinical use.

1.8.1 Buccal mucosa

Buccal mucosa is relatively easy to obtain and is a good source of fibroblasts. We have extensive experience in our laboratory of isolation, culture, attachment of cells to scaffolds and implantation in patients of fibroblasts obtained from buccal mucosal biopsies [256] [255]. Excess mucosal biopsies, from substitution urethroplasty operations (where patients have given informed consent for some of their tissue to be used for research) are easily obtained and will provide a good source of fibroblasts for *in vitro* studies. Barbagli *et al*. describe data from 350 patients who underwent a single cheek biopsy of 4 X 2.5cm [257]. Eighty-five per cent of patients had no pain post-operatively, 73.4% had oral numbness for one week and 98% of patients said they would be happy to undergo the biopsy procedure again. The volume of oral biopsy required, which will be approximately $1cm^2$, for isolating fibroblasts is much less than that taken by Barbagli and colleagues and therefore complications from this aspect of the procedure, if it did reach the clinic, are expected to be even less.

1.8.2 Fibroblasts

Fibroblasts are the most common connective tissue cell. They are responsible for secretion of ECM rich in collagen and other proteins and molecules described above [258]. Inactive fibroblasts are known as fibrocytes. Fibroblasts may be recognised by their branched cytoplasm surrounding an elliptical, speckled nucleus (Fig 1.8.2.1). Migration is a noted feature of fibroblasts. In addition to this they are noted to retain positional memory and if transplanted ectopically, they will retain memory of their previous functional role over a few generations [259]. Fibroblasts may differentiate into myofibroblasts. Myofibroblasts have been observed to express smooth muscle actin and are responsible for contraction [260].

1.9 Cell culture conditions

In our laboratory, we have experience with the culture of fibroblasts on scaffolds for various purposes. The optimum method for fibroblast culture is described in the methods section. This thesis will continue the use of these culture conditions and attempt to progress research on this topic further. From the literature, four studies have cultured fibroblasts on scaffolds used in SUI/POP repair (section 1.6.6). Kapischke *et a*l. and Langer *et al*. used Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal calf serum with unrestrained scaffolds [237] [238]. Skala *et al*. used Dulbecco's Modified Eagle Medium (DMEM) with 5ng/ml platelet derived growth factor (PDGF) and scaffolds restrained in plastic rings [236].

We could not find any published studies utilising chemical mediators or physical regimes to improve the outcomes of tissue engineered prostheses to be utilised in POP/SUI. Syedain and Tranquillo have shown Transformin growth factor- β (TGF-β) and cyclical stretching of fibrin based constructs may be used to stimulate collagen production by fibroblasts and increase ultimate tensile strength/ Young's modulus [261]. In this study, we aim to investigate the use of chemical mediators and physical regimes to improve our tissue engineered prostheses.

1.10 Biomechanical testing

As discussed earlier paravaginal tissue weakness is one of the postulated mechanisms of etiological significance in both SUI and POP. The common forms of treatment aim to correct this weakness by inducing fibrosis and re-strengthening the weakened tissue. Re-enforcement with a weaker substitute is considered to be counterproductive and a stronger less flexible prosthesis may lead to erosion of existing tissue and patient discomfort. Therefore the study of the biomechanical characteristics of paravaginal tissue is central to developing tissues for clinical use.

There are no standardised protocols for testing the biomechanical properties of vaginal tissue. Many researchers have looked solely at structural properties of materials, not taking account of geometric data. For instance performing uniaxial testing to measure breaking load of a tissue plotted against elongation. This methodology takes no account of tissue size; in particular tissue thickness and thus is inappropriate for comparative purposes. More accurate testing should involve calculation of stress (force divided by cross sectional area of tissue) versus strain (change in elongation in relation to initial length) data. The data that may be obtained from this graph (Figure 1.10.1) is:

1) The ultimate tensile strength (UTS)- the maximal stress the tissue may take
2) The ultimate strain (UT strain)- the strain corresponding to the point of maximal stress
3) The tangent/ elastic/ Young's modulus (YM)- the slope of the linear portion of the curve, gives information of ability to stretch
4) The strain energy density- area beneath the curve

Normally the curve is non-linear and has three distinct sections; initial toe, linear and failure regions [262]. In order to calculate the YM the volume of the tested tissue must be calculated. This may be done via contact and non-contact methods [263]. The latter involves the shadow amplitude method, the profile method, and the use of laser technology [264]. The most accurate are non-contact methods as there is less risk of causing weakening of tissue via measurement, although in practice this may be minimal depending upon the tissue being tested. The next question that arises is the method of securing the tissue for uniaxial loading. Some researchers have used clamp grips and others pin grips. The latter we feel is less accurate than clamping both sides of the tissue, as the clamp may purchase the full width of the tissue and distribute the force more evenly across the tissue. Another variable is the strain rate. When studying ligaments and tendons from animals; strain rate has not been shown to affect the biomechanical properties [265] [266].

Much work has gone into looking at the effects of variables of tissue conditions such as ambient air, storage in saline, temperature, and freezing on the biomechanical testing of paravaginal tissue [267] [268]. Rubod *et al*. explored the differences between fresh and frozen samples in terms of the mechanical properties of vaginal tissue, and found that the effect of frozen storage was negligible [267]. The same authors also found vaginal tissue in sheep to be anisotropic, thus implying direction of uniaxial testing may affect results. This can only be resolved by bi-axial straining in two orthogonal axes.

Moreover, living tissues are viscoelastic/ viscoplastic and have the ability to retain stretch and weaken with repetitive strain. An elastic material will return to its original shape when a force or load is removed, without the need for compressive force. If residual deformation is present it is recognised as plasticity. Viscosity in a material is described as an elongation over time due to a sustained load. Most biologic materials will exhibit a mixture of these properties [269]. This behaviour results from the complex interactions of the collagen, elastin, proteoglycans, and water within the tissue. These behaviours are important for processes such as delivery because they allow the tissue to mechanically adapt and soften over time to reduce the likelihood of injury. There are a variety of viscoelastic tests that may be performed [270]. For instance cyclical loading can be used to measure the area between loading and unloading

curves, which describes the viscous phenomenon of energy dissipation, i.e. hysteresis. Pena *et al*. discuss the use of uniaxial stress and relaxation curves to estimate the viscoelastic properties of paravaginal tissues and describe this for a small series of patients with prolapse [271]. The viscoelastic properties have been modelled to show the effect of damage processes and more recently have been used as a model for vaginal tissue [271] [272].

When testing human vaginal tissue, the degree of hydration has been found to affect the tensile properties of the tissue to be tested. Anterior vaginal tissue was removed from 42 patients with a cystocele and tested within two hours of extraction [273]. Factors the authors controlled to prevent influence on tissue strength were temperature and time delay. Comparing tissues immersed in saline, with tissues wrapped in moist gauze the authors found points of pre-transition strain, YM and yield point were significantly lower in the former group, whereas UTS was significantly higher. The authors postulate this is due to water absorption causing swelling and disruption to loose connective tissue reducing YM. There are no standardised criteria for tensiometry testing of recently excised human tissues. However, one would ideally aim to prevent delay of testing to maintain the tissue in its original form. If a delay is inevitable then one must endeavour to maintain tissue sterility and prevent desiccation. Rudob *et al*. found tissue soaked in saline remained stable up to 24 hours and this had no effect on the biomechanics of the tissue, storage in paraffin did not affect the results either [267]. Also when comparing samples at room temperature to samples at 37^0C no differences were found.

Also the extent of stress testing has been questioned. DeLancey has reviewed pelvic floor dimensions and loading [274]. The area of the average female pelvis is $94cm^2$ [275]. The load on the pelvis comes from the abdominal viscera, muscles and from respiratory activity. In the supine position the load is 19N, this rises to 37N in the standing position and reaches 129N on coughing.

Therefore, testing to failure usually to a level much higher than this is thought not to be required and more importantly should not be used for comparison between tissues. Another important consideration is that during parturition the pelvic stretch ratio in the pelvic muscles can reach 3.26 [274]. However it must be born in mind this is at a time when tissues have completely adapted to accept this stretch and may be less relevant in the post menopausal female who will not undergo such strain.

Moreover, until the ideal testing conditions are explored further it seems prudent to keep to a physiological environment and avoid tissue fixation and freezing. At present, data is still lacking

on the uniaxial testing properties of vaginal tissue and more complicated biaxial testing may need to be developed later. Cyclical loading also needs to be developed further for testing vaginal tissue.

1.10.1 Biomechanical testing protocols

There have been three major protocols published in the literature for the biomechanical testing of paravaginal tissues [276], [267], [273].

Ettema *et al*. describe application of high frequency vibrations during slow length uniaxial changes to the tissue. This method was first described when testing contracting skeletal muscle to distinguish contractile from passive properties [277]. The authors claim, the high frequency vibration allows a more detailed analysis of the non-linear relationship between stiffness and stress and allows stiffness to be measured over a wider range of stresses. Tissue in their study was wrapped in saline soaked gauze and frozen to -70°C. The strips were thawed in approximately 1 hour to room temperature and trimmed to a rectangular size (approximately 50mm long, 10mm wide). The ends of the material were sutured and glued in place. The tissue was kept in paraffin oil to prevent desiccation. The stretch rate was kept constant at 0.8mm/s. The experiment was terminated at a peak force of 18N.

Zimmern *et al*. describe a methodology to test freshly collected samples within two hours of harvest. They compared tissue transfer in moist gauze with transfer in saline. Both were kept on a bed of ice to maintain a constant temperature. A 2.2N preload was applied to remove slack. Samples were mounted between aluminium blocks lined with adhesive tape. Uniaxial testing was performed and the sample strain rate was 0.5mm/sec. Stress versus strain was plotted and YM, UTS and “yield point” strain (UT strain) were recorded. It was found hydration weakened the samples and samples kept in moist gauze had a higher UTS and YM [273].

Rubod *et al*. describe uniaxial testing using clamp grips and a constant deformation rate of 0.02mm/sec. A rate of 0.2mm/sec led to few measurements and a slower rate of 0.002mm/sec led to tissue dehydration due to a longer test and this affected the results. The authors found tissue freezing or tissue temperature had no effect on biomechanics but advise that all tissues should be frozen at -18 C in physiological solution and defrosted for nine hours before testing. Furthermore, samples should not be kept in solution longer than 24 hours prior to testing and tissue should also be cut and tested in the same orientation.

1.10.2 Biomechanical properties of paravaginal tissue

Only four studies could be found where the biomechanical properties of paravaginal tissue were investigated [6] [17] [18] [19] (Table 1.10.2.1). These all looked at women with POP. No studies were found where tissue from women with SUI was studied. From these only one study looked at the biomechanical properties of healthy (non prolapsed) paravaginal tissue [19]. The authors found women with POP to have less elastic tissue which tore at lesser stress and strain. The YM and UTS was seen to correlate with the degree of prolapse with a higher grade of prolapse having a higher YM and lower UTS. It was also noted that postmenopausal women had tissue with lower UTS and higher YM. The graphs of the stress vs. strain are not included in the paper, nor is there mention of linearity of data. Also one must comment on the testing protocol where the tissue underwent a freeze-thaw process, thus potentially not giving us accurate data of the tensile properties of vaginal tissue.

Using uniaxial testing at a rate of 0.8mm/s, comparing tissue strength of prolapsed tissue in pre and post-menopausal women Goh found a higher YM in postmenopausal vaginal tissue, this was put down to an age related phenomenon, possibly related to tissue hydration and maturation of collagen crosslinks [269]. Epstien *et al*. found women with a vaginal prolapse had significantly more extensibility in their vaginal skin compared to women without prolapse [246]. The skin laxity was noted to have a linear relationship with severity of prolapse and was not related to a systemic skin laxity. However vaginal skin extensibility could have been a manifestation of the stretch applied to the skin as a result of the prolapse and as the prolapse grade worsens the skin extensibility increases. Although to counteract this, the authors used sidewall vaginal tissue which is the least likely to be affected by stretching from a prolapse and is more likely to represent baseline vaginal tissue. The researchers used a vacuum cutometer MPA to estimate YM, with tissue undergoing cyclical testing.

Paravaginal tissue in women with POP was found to be hyper-elastic with large deformation before rupture [278]. The authors only looked at tissue of five patients with prolapse and compared this to tissue from five cadavers without prolapse. All tissues underwent the freeze thaw process. As the curves were non-linear calculation of YM was not felt to be appropriate. However, looking at the curves we see an initial “toe in” portion followed by a linear region to the curve therefore YM could have been calculated in this region. Moreover, others have successfully calculated YM to define elasticity for this tissue [279] [269] [280] [276].

Intervariability was found between pelvic ligaments and tissues when tested for resistance to tear, however, this was performed on cadaveric tissue and the study only measured the

breaking force required [281]. The iliopectineal ligament was statistically significantly stronger than the sacrospinous and arcus tendineus pelvic fascia varying from 22 to 200N for the break point. The same authors looked at the strength of vaginal tissue in 16 women with prolapse, who were undergoing prolapse repair. The samples were from patients with posterior prolapse and were tested for tensile strength and a second group of samples for break point [282]. To calculate the break point the tissue was held and a piston of 1cm diameter plunged into the tissue measuring distension and force.

We have combined the above data where possible to provide ranges of biomechanical properties observed for paravaginal tissue in both prolapsed and non-prolapsed states. The ranges for pre-menopausal women without prolapse are important in providing us with a baseline to aim for when engineering replacement tissue (Table 1.10.2.1). When replacing damaged vaginal tissue it would seem logical that the biomechanical range of normal tissue should be the target range for prostheses to reduce the risk of failure and erosion. Indeed it has been postulated that prostheses with a decreased UTS will lead to recurrence and those with an increased YM may lead to erosion. In section 1.10.5 we analyse whether this is the case.

1.10.3 Mechanical properties of pelvic organ prostheses

In total we found 15 papers assessing the biomechanical properties of slings/meshes used in POP/SUI [283] [284] [285] [286] [287] [288] [289] [290] [291] [292] [293] [294] [295] [296] [297]. As can be seen from the tables in appendix 1, a variety of materials have undergone uniaxial testing by a variety of methods. Those calculating stress vs. strain data are summarised in table 1.10.3.1. We can clearly see the wide range of biomechanical properties described for the various prostheses. Below, (Section 1.10.5) we correlate these properties to success and complication rates.

Author	Sample	Ultimate tensile strength (MPa)	Ultimate tensile strain	Young's modulus (MPa)
Cosson *et al*. 2004 [296]	Prolapsed vaginal tissue		0.15	
Goh *et al*. 2002 [281]	Premenopausal Prolapsed vaginal tissue			9.5
	Postmenopausal Prolapsed vaginal tissue			11.3
Lei *et al.*	Premenopausal, no pelvic organ prolapse	0.79	0.68	6.65
2006 [297]	Postmenopausal, no pelvic organ prolapse	0.42	0.37	10.26
	Premenopausal, pelvic organ prolapse	0.6	0.5	9.45
	Postmenopausal, pelvic organ prolapse	0.27	0.14	12.1
	Ranges for prolapsed tissue	**0.27-0.6**	**0.14-0.5**	**9.45-12.1**
	Ranges for non-prolapsed tissue	**0.42-0.79**	**0.37-0.68**	**6.65-10.26**

Table 1.10.2.1. Biomechanical properties of paravaginal tissue.

Author	Sample	Ultimate tensile strength (MPa)	Ultimate tensile strain	Young's modulus (MPa)
Hilger *et al*.	Autologus fascia (Rabbit)	1.57		4.67
2006 [298]	Pocine dermis	5.14		26.72
	Porcine collagen-coated polypropylene mesh	4.74		4.53
	Human cadaveric dermal graft	10.68		30.59
Pierce *et al*.	Porcine dermis	2.31	0.55	9.18
2009 [299]	Polypropylene	2.59	0.41	9.04
Moalli *et al*.	Gynecare			3.17
2008 [304]				
(corrected for	Boston Scientific			2.88
thickness)				
	AMS			2.58
	Bard			1.94
	Caldera			2.5
	Mentor			5.6
Atmaca *et al*.	Prolene	25.2	1.81	
2008 [311]	Mersilene	9.2	1.31	
	Ultrapro	2.2	0.91	
	Vypro	8.1	0.84	
	Ranges for prosthesis	**1.57-25.2**	**0.31-1.81**	**1.94-26.72**

Table 1.10.3.1. Biomechanical properties of pelvic organ prostheses.

Author	Sample	Ultimate tensile strength (MPa)	Ultimate tensile strain	Young's modulus (MPa)
Hilger *et al*.	Autologous fascia	1.12	-	3.3
2006 [298]	Porcine dermis 1	0.82	-	4.78
(Post 12 weeks in rabbit abdomen/vagina)	Porcine collagen-coated polypropylene mesh	3.52	-	3.17
	Human cadaveric dermal graft	1.43	-	8.26
Pierce *et al*. **2009 [299]** **(post 9 months**	Porcine dermis 2	1.84	0.35	7.27
in rabbit vagina/ abdomen)	Polypropylene	1.6	0.38	87.48
Walter *et al*. **2003 [309]** **Post explantation at 12weeks from rabbit vagina**	Human cadaveric fascia	2.0	-	8.1
	Range post explantation	**0.82-3.52**	**0.35-0.38**	**3.3-87.84**

Table 1.10.4.1. Biomechanical properties of pelvic organ prostheses post explantation from rabbit vagina

1.10.4 In vivo host response to the mechanical properties of pelvic organ prostheses

The scientific basis for using pelvic organ prostheses is to induce fibrosis in the local tissue allowing for support to the posterior wall of the urethra or walls of the vagina thus reducing the chances of urine leakage or POP recurrence. Therefore some authors have looked at the effect of this tissue reaction on the biomechanical properties of pelvic organ prostheses. In our review, we found three papers which assessed these parameters for prostheses after explantation from the paravaginal tissue [283] [284] [294] (Table 1.10.4.1). All three used a rabbit model. Hilger *et al*. and Walter *et al*. explanted materials at 12 weeks whereas Pierce *et al*. explanted at nine months.

We found all materials underwent a decrease in UTS after implantation in the paravaginal space of rabbits (Fig 1.10.4.1). Rabbit fascia, cadaveric fascia and cross linked porcine dermis (porcine dermis 2) decreased in YM, whereas polypropylene (PPL), cadaveric dermis (CD) and fenestrated porcine dermis (porcine dermis 1) increased and porcine collagen coated PPL remained the same (Fig 1.10.4.2). Cadaveric tissues underwent the greatest decrease in strength which is in keeping with the histological findings of gross degradation and this also explains the clinical findings with these tissues [226] [222]. Fenestrated porcine dermis grafts lost strength and increased in YM, but cross linked porcine dermis did not change a great deal. This may be because the latter is known to become encapsulated and no remodelling occurs within it [214] [298]. These results point to better integration for fenestrated grafts, which is also the case in hernia models [299] .

The biomechanical properties of autologous rabbit fascia were unchanged and this leads us to believe they are not altered significantly by the host response to them [214]. Over time collagen remodelling has been demonstrated in autologous tissue [222]. PPL and porcine collagen coated PPL decreased in UTS. PPL, however, became stiffer on implantation whereas, PPL coated in collagen was resistant to that increase.

Dora *et al*. [300] looked at tissue strength of five materials used as slings, which were transplanted on rabbit rectus fascia. Human cadaveric fascia, porcine SIS & PD all decreased in strength at 12 weeks compared to baseline, whereas PPL mesh and autologous fascia kept their tensile strength [300]. When looking at stiffness of tissue after 12 weeks implantation, human cadaveric fascia, PD and SIS decreased significantly, whereas that of PPL increased and autologous fascia stayed the same. The authors also looked at change in surface area of the

tissue and found; no change in human cadaveric fascia, PD and PPL mesh, whereas SIS decreased by 41% and autologous fascia by 50%.

With the same materials Krambeck *et al*. [301] requested an opinion from an independent pathologist of the degree of fibrosis present in tissue at six and 12 weeks post implantation. They found all tissues demonstrated a high degree of fibrosis, which is noted to be highest with the PPL mesh and least in porcine material. The researchers also looked at the degree of inflammatory infiltrate present on the tissue at six and 12 weeks post implantation finding PPL mesh to have the least B and T cell markers. Thus the authors claim PPL is a superior material by having a low inflammatory infiltrate and greater fibrosis/ scarring at both six and 12 weeks. The major criticism of this is the association of inflammation with weakening of tissues. It is well known tissues will progress through an initial inflammatory process in order for fibrosis/ scarring to occur [302] [303]. The time taken for this to occur will vary between materials. We also know materials may be weakened by the inflammatory process (macrophage activity) prior to remodelling into stronger structures. This remodelling will vary between tissues and may simply occur earlier in the course for PPL. A study looking at a more complete time course would be more appropriate.

Speiss *et al*. [286] compared cadaveric fascia lata to PPL at six and 12 weeks implantation in a rat model. The rats were implanted with both materials, one on each side to reduce variability. The criticism of this study is that the slings were not implanted paravaginally. Other authors have placed pelvic organ prosthesis in abdominal walls of rats, rabbits or pigs to test the effect this would have on the biomechanical properties of the prosthesis [304] [305] [296] [295] [297] [306] [307].

Sergent *et al.* describe a good time course and changes in biomechanical properties up to 180 days in an abdominal wall model [304]. These, although useful, we feel may not provide the same data as vaginal placement as it is hypothesised that the endogenous microflora and increased vascularity of the vagina may lead to increased autolysis of graft material [294].

In conclusion, it is difficult to predict the biomechanical behaviour of prostheses implanted within the human vagina. From animal studies we see weakening occurs early on, followed by some strengthening when fibrosis occurs. Moreover, the experiments of vaginal implantation would need to be repeated with longer periods of implantation to observe the effect of time on the strength of the tissue. These studies may have clinical implications and may help in predicting prosthetic failure and erosion.

1.10.5 Correlation of mechanical properties, host response and success of pelvic organ prostheses

We correlated the mechanical properties of UTS and YM for prostheses, reported in the literature, with those of native tissue (Fig 1.10.5.1).

The materials that are closest to native tissue in terms of UTS and YM are rabbit rectus fascia [283], porcine dermis 2 (cross linked) [284] and macroporous monofilament PPL [284]. The materials with a greater YM and UTS are cadaveric dermis and (fenestrated) porcine dermis 1 [283]. Porcine collagen coated PPL has a slightly lower YM but a much greater UTS than native tissue [283].

When matching the properties of UTS and YM to success and erosion rates we found the materials closest to native tissue had varying success and erosion rates. Macroporous PPL has been shown to have good success rates with SUI and POP repair but has a greater risk of erosion [308] [309] [310] [311]. At five year follow up, in a randomised trial, Tate *et al.* describe a better objective success rate with PPL mesh compared to autologous fascia lata [312]. There are no reports of rabbit rectus fascia being used clinically. The success of cross linked porcine dermis is very low but the erosion rate is also low[137]. Natale *et al.* compared PPL mesh with PD mesh and found an objective cure rate of 71.9% with PPL and 56.4% with PD [313]. The erosion rate was 6.3% and 0% respectively.

To our knowledge, fenestrated PD has only been described in one study in the literature for use in POP or SUI [314]. The results of early follow up in 35 patients were good with no complications but the number of patients so far studied is too low as yet to draw any meaningful conclusions. The other material with much greater YM and UTS than native tissue was cadaveric dermis. At medium term follow up, of 6-12 months, cadaveric dermal grafts showed high failure rates of 50% [227] [228]. Erosion rates varied between 0% [180] [231] to 11% [232] [315]. The material with much higher UTS and lower YM was porcine collagen coated PPL mesh. This is currently undergoing clinical testing and at present no data could be found for either its success or complication rates, clearly these results need to be assessed in future years.

From the above evaluation of the current studies we were unable to demonstrate any simple correlation between the biomechanical properties of prostheses and the success and complications reported for them. It is likely that other factors will influence the final outcome. The host response to implanted materials is acknowledged to be at first sight very variable and this is discussed next.

The relationship of the biomechanical properties of prostheses post explantation to native tissue are shown in Figure 1.10.5.2. There are no prostheses post explantation that have similar UTS and YM to native tissue. Cadaveric dermis, cadaveric fascia and PD had similar YM to native tissue, whereas rabbit rectus fascia and fenestrated PD had similar UTS but lower YM. Porcine collagen coated PPL was a lot stronger and less stretchy, whereas PPL was both stronger and less stretchy.

We see that PPL post explantation has extremely high YM and has high UTS. This may explain its greater risk of causing erosion. We note that rabbit rectus facia, porcine collagen coated PPL and fenestrated PD are stretchier than native tissue (lower YM), from these only the fenestrated PD has been used clinically but only in one study [314]. It would be interesting to see the clinical findings of porcine collagen coated PPL, however rabbit studies revealed it to have caused erosion in two out of two rabbits after 12 weeks of implantation [283]. Cadaveric dermis, cadaveric fascia and PD have the same stretchiness as native tissue but a higher UTS, this again does not correlate with their clinical findings.

1.10.6 Properties of materials to be tested

We aim to investigate seven materials to produce tissue engineered prostheses. The materials we selected were Alloderm (AL), cadaveric dermis (CD), porcine dermis (PD), porcine small intestinal submucosa (SIS) and polypropylene (PPL) which have all been used clinically in SUI/POP with variable results as discussed below. We will also investigate two new materials not used in SUI/ POP surgery; sheep forestomach (SF) and thermoannealed poly(L)lactic acid (Th PLA). This is because these materials have been shown to be good in tissue engineering applications as discussed below.

1.10.6.1 Alloderm (AL) and Cadaveric dermis (CD)

CD has its epithelium and all cellular components removed leaving behind an acellular biological matrix. These materials have been used in both SUI/ POP surgery and tissue engineering and were thus selected on this basis. The different companies manufacturing CD have patented processes for cellular removal. The dermis may be dried in various ways including freeze or solvent. AL is a specific type of CD manufactured by LifeCell Corporation. Lemer *et al*. found only small differences in maximum load and stiffness between the different types of drying processes [304]. Also to increase tensile strength the biological matrix may be cross linked. The tensile properties of CD have been described by three studies [287] [296] [304].

Choe *et al*. have described AL as having a maximum load of 60% of human fascia lata but to stretch twice as much before failure [300]. Hilger *et al*. found implantation in a rabbit abdomen/vagina for 12 weeks led to a significant decrease in UTS (seven fold) and YM (three fold) [296]. This is in keeping with the expected inflammatory response and clinical observations. Conversely when placed in a rat abdomen, AL was found to increase in UTS at both 30 and 60 days [316]. However, it must be noted, the authors explanted graft with adjoining abdominal wall, which could have led to its increased strength. Collagen deposition and neovascularisation was higher with AL than vicryl.

Different cadaveric fascia meshes have been shown to induce different inflammatory markers *in vitro*. This includes monocytes, macrophages, interleukins and VEGF [317]. Sclafani *et al*. have shown good fibroblast infiltration of CD from biopsies taken from human subjects implanted with it in the skin behind the ear [318]. However, in vitro, Ng *et al*. found fibroblasts proliferated around the periphery of CD grafts with limited penetration into the matrix [319]. In this study, CD did allow for collagen production. CD has also been found to form a capsule

around it with minimal cell infiltration in a rat abdominal model [320]. The authors also found CD to induce more inflammatory cells than fibroblasts (low aspect ratio) compared to SIS.

Blander *et al*. performed histological examination of five cadaveric fascia slings removed at re-operation [321]. The slings demonstrated collagen but lacked any cellularity. Similarly Woodruff *et al.* removed slings from three patients with failed procedures and found CD slings only to be infiltrated peripherally [221]. They also had an element of degradation. Clinically CD grafts used for POP or SUI have had high failure rates especially at medium term (>1 year) follow up [322] [231]. This is in keeping with the *in vitro* findings above.

1.10.6.2 Polypropylene (PPL)

Polypropylene is the most common synthetic material in use for the surgical treatment of SUI/ POP and therefore we included it in this study. Accordingly, various combinations with other materials exist along with different patterns of weave and manufacture. As described earlier, Amid classified synthetic meshes into 4 categories [209]. Type 1 macroporous PPL has been recommended by NICE (guideline CG40). Therefore we will investigate this for its potential use in tissue engineering applications.

In the literature, eight papers were found which reviewed the tensile properties of type 1 PPL mesh used for SUI/ POP surgery [284] [286] [287] [288] [289] [290] [292] [293]. Pierce *et al*. showed PPL to weaken and become stiffer with implantation in a rabbit vagina/abdomen after nine months [284]. Weakening was also the case when implanted in a rat abdomen for six and 12 weeks [286]. The only comparable results, reporting stress vs. strain data, were from Pierce *et al*. where the UTS of PPL was found to be 2.59MPa and the YM 9.04MPa [284]. The other researchers did not take account of tissue size and so cannot be compared, however, different types of PPL within the same study showed different biomechanical profiles.

Skala *et al*. attached vaginal fibroblasts to seven different meshes and found xenograft meshes allowed for the best attachment of vaginal fibroblasts [236]. However Obtape (thermo-annealed PPL) also allowed good fibroblast attachment at both five days and five weeks *in vitro*. This was measured by a semi-quantitative method using immunofluorescence microscopy. Macroporous PPL meshes had poor coating of fibroblasts. The fibroblasts coated along the filaments but could not bridge the pores. The prostheses with smaller pores (75-50µm) had better fibroblast coating. Slack *et al*. noted more of an inflammatory response with microporous tapes (IVS and Obtape) compared to macroporous (Monarc) [323]. The Monarc was better integrated and showed more collagenous formation. The microporous meshes

showed more inflammatory cells, less fibrous tissue and severely restricted formation of collagenous bridges through the meshes.

Kapischke *et al*. have precoated PPL meshes with human foreskin fibroblasts and used scanning electron microscopy (SEM) to show that fibroblasts settle on PPL threads [238]. Fibroblasts also showed variability by developing long extensions and a ruffled surface. The meshes were also seen, by immunostaining, to have collagen I and III. Similarly Langer *et al*. showed fibroblasts may coat material surfaces. They found fibroblasts prefer to grow on low-weight meshes, thin filaments and mesh nodes [237]. A microporous mesh had better coating than a macroporous mesh. Interestingly, when looking at Vypro mesh (PPL and polyglactic acid combined) at six weeks when both were present fibroblast attachment was good, however as the polyglactin dissolved at three months the fibroblast attachment was 50% of that at six weeks.

Pierce *et al.* studied the inflammatory response of rabbit vaginal tissue with PPL mesh [324]. PPL showed less inflammation but greater fibroblast proliferation compared to PD. Neovascularisation was present in both meshes. Erosion was 27% and 15% respectively. The inflammatory response with PPL was milder, more uniform and showed good tissue integration consisting of new collagen, fibroblasts, blood vessels and smooth muscle. Elmer *et al*. took pre and post operative biopsies of women undergoing pelvic reconstructive surgery with PPL mesh one year after placement [325]. Patients with mesh at one year showed a reduction in fibroblasts but an increase in macrophages and mast cells. This suggests a mild inflammatory but enduring foreign body cellular response. This is in keeping with animal studies [326] [324]. The reduced number of fibroblasts may be due to the timing of the biopsy and at 1 year the need for fibroblasts may have passed if structured tissue had been formed. There were no significant differences in collagen density. Three cases from 10 showed mild granuloma formation and two showed mild erosion.

Clinically, TVT (lightweight PPL) has good success rates for the treatment of SUI. Objective and subjective cure rates of 80% at seven years are recorded [327] [328]. Liapis *et al.* reported complications at seven years to involve 11% of patients with detrusor overactivity, 19.6% with urgency and one patient out of 65 with vaginal erosion [327]. Meta-analysis by Jia *et al*, involving 10 RCTs consisting of 1148 patients found objective recurrence was halved by the use of a mesh from 30% to 14% [183]. PPL had a failure rate of 9% at a median term follow up of 13 months. Monofilament PPL mesh has been found to have an erosion rate of 10.2% when used in the treatment of POP [218]. All erosions in this study were evident by the first clinic

visit. Foon *et al*. described an erosion rate of 14% in their meta-analysis with the use of PPL [184].

In summary Type 1 macroporous PPL comes in many different packages. It has good results in the treatment of SUI over a seven year follow up period. Its use in the surgical treatment of POP has no long term data. Short term data suggests it to be better than no mesh but does have a high risk of erosion. Attempts to attach fibroblasts have shown mixed results; meshes with smaller pores having greater success. Nevertheless fibroblasts do attach to the macroporous PPL and allow for an inflammatory response. The expected inflammatory response with macroporous PPL mesh is one of a prolonged low grade foreign body cellular reaction, with occasional mild granuloma.

1.10.6.3 Porcine dermis (PD)

PD is a decellularised porcine skin and it may be cross linked to make it less likely to be digested by collagensaes. It has been used for tissue engineering and in a few clinical studies described below. Its mechanical properties have been described in two studies, Hilger *et al*. used a fenestrated PD [283] and Pierce *et al*. describe a cross linked PD [284]. As discussed above in 1.10.5 cross linked PD is closer to native tissue in its mechanical properties but fenestrated PD allows better integration. In a rat hernia model cross linked PD was shown to be more durable up to one year and had greater UTS [329]. In addition, cross-linking may occur during sterilising protocols and in our experience ethylene oxide [330] and paracetic acid [331] both decreased dermal pliability when used to sterilise human donor dermis. Recent thinking in this area suggests that scaffolds based on natural matrices which are strongly crosslinked (for example with glutarldehyde) to the extent that the host cells cannot break them down will provoke a chronic inflammatory response [332], often ending up with encapsulation and failure of the implant. Badylak discusses the process of remodelling by the immune system of modified natural scaffolds [333]. Mantovani *et al*. [334] point out that macrophages are capable of responding to materials (living and non-living) introduced into the body by acquiring a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. Following on from this is the premise that any scaffold to be implanted must be capable of being remodelled and replaced by the body's own ECM.

Woodruff *et al*. have found PD to be encapsulated upon explantation from four women requiring revision of their sling surgery [214]. Ghandi *et al*. found variable host responses including foreign body reaction, encapsulation and inflammation in 12 patients requiring re-operation after a PD graft [298]. At a mean follow up of 24 months, cure rates from anterior vaginal repair with PD were shown to be 78% [335]. However, 17% of patients receiving a PD

developed epithelial sloughing resulting in vaginal discharge presumably due to placement of vaginal mucosa over an avascular graft. Gomelsky *et al*. have shown 7% of 240 women developed vaginal extrusion of PD grafts after pelvic organ surgery [336]. Leboeuf *et al*. have reported a 7% recurrence rate without significant complications at 24 months [337]. In conclusion, although the early results with PD have been good, 36 months follow up has shown success in only 54% of patients after PD sling surgery for SUI [137].

1.10.6.4 Sheep forestomach (SF)

Sheep forestomach is a decellularised extracellular matrix proposed to have good biophysical characteristics and been shown to attach cells well [338]. It has also been shown to allow the growth of human fibroblasts and keratinocytes [339]. It has however, not been utilised in the treatment of SUI/ POP.

1.10.6.5 Porcine small intestinal submucosa (SIS)

SIS is a porcine-derived graft obtained from the small intestine submucosa (submucosa, muscularis mucos and stratum compactum). The graft is acellular and composed of non-crosslinked collagen (types I, III, and V), glycosaminoglycans, proteoglycans, glycoproteins, and multiple growth factors. The extracellular matrix also contains cytokines, binding proteins, growth factors and matrix receptors that regulate cellular migration, infiltration, proliferation and differentiation [340] [341]. SIS is reported to be replaced by 120 days [342]. It is a commonly used material in tissue engineering applications and has been used cell free in pelvic floor surgery.

Rice *et al*. have described an increase in UTS of SIS when implanted in a rat abdomen [316]. However excision of the graft included adjacent abdominal wall tissue potentially confounding the results. Collagen deposition and neovascularisation was higher with SIS than with vicryl. SIS has been found to have near complete cellular infiltration with minimal encapsulation when implanted in a rat abdomen [320]. The authors have also found a high aspect ratio described as; increased fibroblasts compared to inflammatory cells. This is important as inflammatory cells are required initially but fibroblasts are then required to continue strengthening tissue and produce collagen matrix, to act as a scaffold for new tissue formation. This allows angiogenesis to take place.

Skala *et al*. have shown SIS to act as a good scaffold allowing fibroblast attachment, distribution and proliferation at five hours and five weeks after seeding [236]. Also Cannon *et al*. have described the attachment of muscle derived stem cells on SIS scaffolds confirming its

propensity to allow cellular attachment and distribution [240]. Compared to CD, PD and cadaveric fascia lata, VandeVord *et al*. found SIS to have better biocompatibility allowing increased collagen deposition and angiogenesis[320].

Clinically, in some cases, SIS has been noted to cause an intense inflammatory reaction when used as a sling to treat SUI [343]. Five of 16 patients complained of suprapubic pain and one patient also had induration of the mons pubis requiring surgical drainage. An excessive inflammatory response was also noted by other researchers [344]. However, other authors have noted better clinical outcomes from SIS [342]. Success rates of 93% with 50% of patients having frequency/urgency are reported. Weidemann *et al*. took biopsies from patients with SIS slings and no evidence of a significant immunological reaction or foreign body reaction was noted [345].

1.10.6.6 Thermoannealed Poly-L-Lactic acid (Th PLA)

There are several methods utilised to fabricate highly porous biodegradable polymer cell scaffolds; including particulate-leaching, fibre extrusion and bonding, phase separation, gas foaming, emulsion freeze drying , 3-D printing techniques and electrospinning [346] [347] [348] [349] [350] [351]. Critical determinants for cell attachment are dependent on choice of polymer, fabrication method and scaffold morphology [352] [353]. Electrospinning has emerged as an efficient method of producing scaffolds allowing variation of fibre diameter, porosity and arrangement. These scaffolds may be produced to have a high surface area to volume ratio to provide more surface for cell attachment [354].

Poly(L)lactic acid (PLA)/ Poly(L)-lacto-co-glycolic acid (PLGA) are biocompatible, biodegradable hydrophobic polymers, which have been used clinically for a variety of purposes ranging from bone [355], skin [356], bladder [357], nerve guide conduits [358], adipose tissue [359] and cartilage [360]. The ratio of lactic acid to glycolic acid may be varied. It can easily be moulded into shapes which have good mechanical properties [361]. PLGA lacks chemical moieties that allow cellular interactions and surface chemistries to occur [362]. When different fibre diameters for PLGA were compared ranging from 150 to 6 000 nm, all fibres had a tensile modulus close to normal human skin [351]. The average pore diameters were between 10-14 microns. Human skin fibroblasts attached to these showed significant progressive growth on fibre matrices in the 350-1,100 nm range.

The average half-life of PLGA scaffolds is 10 weeks [363]. PLGA is hydrolysed into lactic and glycolic acids and is metabolised *in vivo* [364]. PLGA as a polymer has been found to degrade faster than pure PLA and slower than pure Polyglycolic acid (PGA) [365]. The degradation

characteristics may be varied by adjusting molecular structure, crystillinity and L/D forms ratios [366]. Also as the fraction of PGA increases the fibre diameter increases [367]. Prior work in our laboratory with polymer electrospinning has produced non-woven randomly arranged fibres with an average diameter of 2.1±1.2 mm for PLA (mean±SD), 2.9 ±1.3 mm for PLA plus lactide oligomers, 2.7 ±0.5 mm for PLGA 85:15 scaffolds, 4.5 ±1.4mm for PLGA 75:25, and 4.3 ±1.5 mm for PLGA 50:50. *In vitro* the PLGA 85:15 scaffold appeared not to undergo any significant degradation over 108 days, whereas the PLGA 75:25 began degradation at 80 days. In contrast the PLGA 50:50 underwent complete degradation by day 24 [367]. Accordingly, it should be possible to design synthetic electrospun scaffolds composed of biodegradable/ bioresorbable materials which can act as temporary tissue supports and guides, providing these can ultimately be replaced in a process of constructive remodelling. This has been the success of these polymers.

De Tayrac *et al*. looked at the *in vivo* degradation of PLA(94) mesh (94% L-lactyl and 6% D-lactyl) and found the mesh retained an acceptable strength for eight months [368]. The *in vivo* response to PLA(94) implantation in a rat abdominal wall showed the inflammatory response was significantly less with the PLA as compared to PPL, and collagen production also showed greater organisation in the former [369]. The authors also found gamma sterilisation increased mesh degradation and decreased UTS making hernias more likely to recur. In a previous study the mesh shrinkage seen with PLA was 23% compared to 24% with PPL, at 90 days, and PLGA (90:10) had completely degraded by 90 days [370]. The tensile strength and strain post explantation of PLA was always higher than PPL at all time points up to 90 days. The same authors also reported PLA to have less infection risk as compared to other meshes in a rat infected abdominal model [371].

In our laboratory, we have found Th PLA has shown a higher UTS (three fold) and YM (50%) than PLA without affecting cellular attachment (unpublished data). This change in properties occurs due to β crystal formation due to the annealing process [372]. This also leads to a reduction in porosity affecting cell viability and surface interactions [373]. Therefore we will assess Th PLA in this thesis. Neither PLA nor Th PLA have been used clinically in the treatment of SUI/ POP.

1.11 Paravaginal tissue histology

Paravaginal connective tissue “endopelvic fascia” is made up of a complex of fibroblasts, myofibroblasts, smooth muscle cells and ECM containing collagen, elastin, proteoglycans, soluble proteins, glycoproteins, fibronectins, and glycosaminoglycans (GAGs) [374]. Resident

fibroblasts are responsible for the synthesis of the ECM and thus endow the tissue with their biomechanical properties. This in turn is mediated by specific cell receptors and cell binding epitopes which play an important role in cell attachment, migration and differentiation. The most dominant component of the ECM is collagen. More than 20 different collagens have been discovered in human tissue. Collagens are proteins forming a characteristic triple helix of three polypeptide chains, which further combine with other supporting proteins to form supramolecular organisations [375]. Collagens may be further subdivided into six groups:

1. Fibril forming collagens
2. Fibril associated collagens (FACIT)
3. Network forming collagens
4. Anchoring collagens
5. Transmembrane collagens
6. Basement membrane collagens

The majority of collagen in connective tissue is fibril forming (types I and III) and provides most of the mechanical strength of these tissues [376]. Type III collagen is abundant in elastic tissues and contributes to mixed fibrils with type I collagen [377]. Type IV collagens provide flexibility and are restricted to basement membranes [378].

Collagen assembly depends on cell type and genetic factors and is controlled by growth factors and cytokines [379]. Most collagen genes reveal a complex extron-intron pattern with the mRNA of fibrillar collagens encoded by more than 50 exons. Mature mRNA is translated at the rough endoplasmic reticulum forming pre-procollagen molecules. Following removal of a single peptide the procollagen molecule undergoes multiple post translational modifications [374]. Hydroxylation of proline and lysine residues occurs at variable points allowing cross-linking [380]. Thereafter the molecules are packaged within the golgi apparatus and secreted into the extracellular space. The organisation of collagen fibrils is contained within the structure, as these align independently and form further covalent bonds. The efficient formation and folding of procollagen chains requires further enzymes and collagen specific chaperones e.g. HSP47 [381].

Collagens are resistant to digestion by proteases [382] and can only be degraded by specific collagenases. Three collagensases have been described. Collagenase A (MMP-1) is the interstitial collagenase. Collagenase B (MMP-8) is mainly specific to neutrophils. Collagenase C (MMP-13) is involved in remodelling. It cleaves type II collagen more efficiently than types I and III. In tissues a balance is found between the opposing actions of collagenases and tissue inhibitors of matrix metalloproteinases (TIMPs).

The endopelvic fascia forms the adventitial layer lying between the vagina and corresponding organs (Fig 1.11.1). The endopelvic fascia is not uniform and may be thicker in certain areas. Although surgeons commonly use this endopelvic fascia for plication when performing a prolapse repair anatomists have often debated whether it exists as a separate structure to the adventitia or is in fact the same thing. The current thinking is that it is the same structure, created by surgical dissection, composed of moderately dense connective tissue with smooth muscle, areolar tissue and loose connective tissue (Fig 1.11.2) [383] [384].

1.11.1 Collagen in POP/SUI

Some authors have found a decrease in overall collagen content of tissue from women with SUI/ POP [71] [72] [73] [74], whereas others have shown a specific decrease in type I [385] [386] and type III [387] [388] [389] collagen using immunohistochemical techniques (Figs 1.11.1.1 and 1.11.1.2). Other reports, on the other hand, have shown no difference in collagen content between women with and without POP or SUI [390] [389] [391]. We know POP and SUI are of multifactorial aetiology and this may explain findings of decreased collagen in certain patients and not others. Also Bailey's review concluded increasing age is associated with changes in collagen and this may be a big confounding factor [392]. Another finding described by Trabucco *et al*. was a non homogenous pattern of staining for collagen with some areas of intense staining and some absent areas of staining in incontinent women but a more homogenous staining in controls. This could lead to sampling error depending upon the location and number of samples taken in each study and may explain the variations of reports in the literature [385].

We know the tensile strength in these tissues is determined by the amount of collagen I compared to collagen III and IV [66]. An increase in collagen I leads to an increase in the UTS of the tissue, conversely excess collagen III and IV decrease mechanical integrity [67]. Menopause seems to decrease the collagen I/III ratio [68] [69]. Falconer *et al*. compared the ultrastructure of paraurethral tissue from postmenopausal women with SUI and from normal controls. The collagen fibril diameter was 76nm in the SUI group compared to 58nm in the control group [112]. The former group also contained a greater concentration of collagen and mRNA expression for collagen I and III with greater cross linking of collagen fibrils. It has been shown that type I collagen contains larger diameter fibrils than type III collagen and the addition of type III decreases collagen diameter [393]. Lang *et al*. have also described an increase in collagen fibril diameter in patients with SUI and POP [394].It is known cross-linking is the last

step in collagen biosynthesis and this greatly increases tensile strength. The other important factor for tensile strength is the fibre length to diameter ratio [395].

In post-menopausal women with SUI the picture is less clear, with some studies showing a decrease[387] [396] and others no change to collagen content [112]. However, greater cross-linking of fibres was observed in these incontinent women, which decreases the flexibility of these tissues [330]. In this study the change was not seen in women undergoing oestrogen therapy.

Collagen I and III levels in women with SUI/POP have been found to be the same or reduced compared to normal women and the mRNA for them has been found to be increased [397]. This suggests a problem with translation, formation of quaternary structure or increased breakdown of collagen. One study showed however that the rate of collagen synthesis was similar in fibroblasts cultured from samples from women with or without SUI [398], implying reduced collagen content may be related to increased breakdown [399]. We have summarised the changes in collagen content and collagen mRNA in women with SUI/ POP as reported in the literature in Tables 1.11.7.1 and 1.11.7.2.

1.11.2 Elastin in POP/SUI

Immunohistochemical staining using Weigert Van Geison staining revealed elastin made up 3.81% and 5.93% of the cross sectional area of endopelvic tissue from women with SUI and a control group respectively (Fig 1.11.2.1) [390]. Elastin provides the tissues with passive recoil after stretch. Elastin sequences interact with multiple proteins found in microfibrils and crosslinking contributes to tissue structural integrity [400]. Elastin has been found to be significantly diminished in patients with POP [75] [401] [402], whereas other studies have found no difference in elastin [389] [403] (Table 1.11.7.1). Elastin expression has been negatively correlated with the degree of prolapse, with tissue from women with greater grades of prolapse showing less elastin [401]. Elastin mRNA has only been described in one study, where it was found to be decreased [404]. SUI is also associated with remodelling of the tissue with respect to elastin-collagen interaction [405]. In addition to this altered elastin metabolism and gene expression have been found in women with POP [70]. Elastase activity is also increased in pelvic floor tissue from women with SUI [406]. Elastic fibre width has been seen to be halved in patients with prolapse [402]. Yamamoto *et al.* have shown elastin mRNA and elastin synthesis was decreased in fibroblasts from prolapsed uterine ligaments compared to non-prolapsed uterine ligaments [70].

1.11.3 Glycosaminoglycans (GAG) in POP/SUI

There are many types of GAG: chondroitin sulphate, dermatan sulphate, heparin sulphate, keratin sulphate, hyaluronic acid and heparin. Dermatan sulphate has been found to be the predominant GAG in paravaginal tissue (85%) [407]. GAGs were found to be increased in patients with POP or SUI [407]. In another study GAGs were not found to have been changed in patients with SUI [408] and another reported decrease in GAGs [409]. Post menopausal women have been found to have decreased sulphated GAGs, dermatan sulphate and chondroitin sulphate [410].

1.11.4 Proteoglycans in POP/SUI

Small proteoglycans such as decorin and fibromodulin bind to different portions of the collagen fibril and are important for fibrillogenesis, whereas other proteoglycans such as biglycan and versican keep collagen molecules apart [411]. Women with SUI have been shown to have less small proteoglycan content than women without SUI [412]. Fibromodulin has been found to be increased [385] and decreased [413] in POP/SUI, whereas its mRNA was found to be decreased [391] [413] or normal [414]. Wen *et al*. found concentrations of prostaglandin to vary according to the woman's menstrual cycle [413]. Decorin mRNA expression was significantly increased in women with SUI and/or POP [387] [413].

1.11.5 Other proteins in POP/SUI

Elastic fibre protein Fibrillin-1 mRNA expression was found to be decreased in patients with SUI [414] but the amount of Fibrillin 1 was found to be normal [391]. Fibrillin-5 mRNA expression was found to be the same [414] between continent and incontinent women, whereas in women with POP it was found to be decreased [415]. Li *et al*. however found fibrillin-5 expression to be significantly reduced in women with prolapse which correlated with degree of prolapse [401]. Fibulin-5 is another protein involved in elastic fibre assembly. Fibulin-5 mRNA has been found to be increased in ligaments of women with POP [416]. Drewes *et al*. have shown fibulin-5 knock out mice to have developed POP [417]. Work from the same group has also shown vaginal protease activity to precede the development of POP [418].

1.11.6 Collagenase in POP/SUI

Studies have revealed increased MMP activity in POP [403]. No difference was found in proMMP-2, MMP-2 or proMMP-9, however MMP-9 was increased in tissue from women with prolapse [419]. Strinic *et al*. found MMP-1 to be increased in uterosacral ligaments of women with POP but not MMP-2 [420], the converse was true in a similar study by Gabriel *et al*. [421]. It must be noted MMP-2 and MMP-9 are gelatinases. Rechberger *et al*. found no increase in collagenase activity in tissue from incontinent women [73]. Contrary to this, Zhang *et al*. found an increase in MMP-9 mRNA expression and a decrease in TIMP-1 mRNA expression in patients with SUI compared with continent controls [422].

Chen *et al*. found para-urethral tissue from incontinent women expressed less tissue inhibitors of metalloproteinases (TIMPs) compared to tissue from continent women [244]. The expression of active metalloproteinase protein was the same. The authors also showed expression of TIMPs may be increased by the use of oestrodiol from cultured fibroblasts from continent women but not from incontinent women [244]. These results imply a lack of TIMP production by fibroblasts in paraurethral tissue of incontinent women, which leads to a pro collagenolytic state in these women.

1.11.7 Paravaginal tissue in SUI/ POP

From the above conflicting data it is unclear exactly what histological changes occur in the tissue of women with SUI/ POP. Generally the studies can be separated to those assessing the amount of each component via staining methods and those assessing component mRNA via real time polymerase chain reaction. We have summarised the studies showing an increase, decrease or no change in the various tissue components (Table 1.11.7.1) and mRNA (Table 1.11.7.2).

The differences in the studies may be due to sampling bias, sample sizes and the use of “controls”. As discussed by Trubacco, non homogenous staining was identified in women with SUI [385]. In the assessment of tissue components 728 patients including controls were assessed. The study size ranged from18-94, average =48.5. In the assessment of mRNA, 480 women were assessed, average study size was 53.3 (range 29-120). Samples from different paravaginal regions were selected, including the endopelvic fascia, vaginal wall, uterosacral ligaments and arcus tendinius tissue. It has been shown that the menstrual cycle may affect the ECM composition and this was not accounted for in these studies [423] [424]. Some

authors have suggested that only women in the proliferative phase of the menstrual cycle should be assessed to standardise this variable [425]. In addition, other contributory factors were not standardised in patients and controls such as weight, smoking and parity, thus potentially leading to the variations in the findings.

Component	Decreased	No change	Increased
Collagen I	[385] [386]	[390] [389] [391]	
Collagen III	[387] [388] [389]	[390] [391]	
Collagen IV		[389]	
Elastin	[426] [401] [402]	[389] [403]	
Glycosaminoglycans	[409]	[408]	[407]
Small proteoglycans			
Decorin	[391]		[413]
Lumican		[385]	
Fibromodulin	[413]		[385]
Large proteoglycans			
Biglycan			[413]
Other proteins			
Fibrillin-1		[391]	
Fibrillin-5	[401]		

Table 1.11.7.1. A summary of the evidence reporting the changes in connective tissue components in women with SUI/POP compared to women without SUI/POP.

Component	Decreased	No change	Increased
Collagen I			[397]
Collagen III			[397]
Collagen IV			
Elastin	[404]		
Glycosaminoglycans			
Small proteoglycans			
Decorin	[391]	[427]	[387] [413]
Lumican	[391]	[414]	
Fibromodulin	[391] [413]	[414]	
Large proteoglycans			
Biglycan		[413] [427]	
Other proteins			
Fibrillin-1	[414]		
Fibrillin-5	[415]	[414]	
Fibullin-5	[391]		[416]

Table 1.11.7.2. A summary of the evidence reporting the changes in mRNA of connective tissue components in women with SUI/POP compared to women without SUI/POP.

1.12 Aims, objectives and hypotheses

The aim is to develop a fibrous connective tissue sheet based on the patient's own oral fibroblasts and either a synthetic or natural scaffold material to improve the quality of repairs that can be offered to women with stress incontinence and vaginal or uterine prolapse.

It is proposed to use autologous fibroblasts taken from a small biopsy of oral mucosa obtained under local anaesthetic, expand these fibroblasts in the laboratory and then introduce them into sheets of candidate materials to make a sheet of connective tissue which can survive long term, will become vascularised post-implantation, and will undergo contraction rather than relaxation when introduced into the body.

The project will:

1. Evaluate two synthetic and five biological support materials as substrates for producing an autologous prosthesis. Candidate materials will be (a) Alloderm, (b) human acellular cadaveric dermis, (c) macroporous polypropylene, (d) porcine dermis, (e) sheep forestomach, (f) porcine small intestinal submucosa, and (g) thermoannealed poly(L)lactic acid. These materials will be combined with oral fibroblasts and assessed for their:
    - Ability to support fibroblast attachment and growth
    - Mechanical properties with and without cultured cells
    - Extent of cell based contraction
    - Ability to support new extracellular matrix production by the attached fibroblasts

2. Assess the effect of scaffold restraint on all of the above properties of the scaffolds.

3. Evaluate the use of Vitamin C for the improvement of tissue engineered materials.

4. Commence work towards the development of a variable stress rig to examine to what extent mechanical conditioning improves the quality of tissue engineered tissues for clinical use.

At the start of this thesis, based on the preceding literature, the hypotheses that can be proposed are that:

1) All scaffolds will support fibroblast attachment and subsequent performance to some extent but that it should be possible to select the most promising to take forward.
2) The mechanical properties of scaffolds will not be significantly affected by the attachment of cells.
3) The mechanical properties of polypropylene, porcine dermis, sheep forestomach and thermoannealed poly(L)lactic acid will be closest to those of native tissue.
4) The extent of cell based contraction will relate to the stiffness of the scaffolds.
5) The biological scaffolds will facilitate the production of collagen and extracellular matrix significantly more than the synthetic scaffolds.
6) Scaffold restraint will have no significant impact on the integration of cells with scaffolds. Restraint will however, lead to increased collagen production.
7) The inclusion of vitamin C will enhance matrix production by cells in the scaffolds.
8) Mechanical conditioning of cells in scaffolds will affect the ECM proteins produced by the cells as a response to the variable stress applied to the materials and cells.

Chapter 2 Methods

2.1 Materials

2.1.1 Alloderm® (AL)

AL was obtained, as a kind donation, from LifeCell Corporation® (Branchburg, New Jersey).

2.1.2 Cadaveric dermis (CD)

Sterile acellular Euroskin® was purchased from the European tissue bank (Beverwijk, Netherlands). This came in glycerol and, as per manufacturer's recommendations, was washed with PBS. Subsequently 1M NaCl was added and incubated overnight at 37^0C. Thereafter the epidermis was removed by scraping with a scalpel handle. The resulting de-epithelialised dermis was then used for experiments.

2.1.3 Polypropylene (PPL)

Sterile macroporous PPL samples were obtained, as a kind donation, from Gynecare Ethicon® (Somerville, New Jersey).

2.1.4 Porcine dermis (PD)

PD was obtained, as a kind donation, from LifeCell Corporation® (Branchburg, New Jersey).

2.1.5 Sheep forestomach (SF)

SF was obtained, as a kind donation from Mesynthes Ltd, (Lower Hutt, New Zealand).

2.1.6 Small intestinal submucosa (SIS)

SIS was obtained, as a kind donation, from Cook medical®. The SIS sheets were 4-ply, acellular and not chemically cross linked.

2.1.7 Poly(L) lactic acid (PLA)

Sterile PLA, as used in chapter 9, was produced in the laboratory clean rooms via electrospinning [367]. Poly(L-lactide) (Mn~99K) was dissolved in dichloromethane (PLA 20% wt/wt) overnight at room temperature from Sigma-Aldrich,Dorset U.K. The fibres were spun in a random orientation from four 2ml blunt tip syringes charged to 17 000 volts (Genvolt UK) at a flow rate of 30µl/min. The fibres were collected on an earthed rotating aluminium cylinder coated in aluminium foil rotating at 200rpm 20cm from the rotator.

2.1.8 Thermoannealed poly(L) lactic acid (Th PLA)

Due to deficiencies in the mechanical properties of PLA noticed in chapter 9, we heat annealed PLA at 60^0C for three hours. This led to an improvement in mechanical properties as shown in chapter 4. The heat annealing method was based on unpublished data in the laboratory.

2.2 Cell isolation and culture

Oral mucosa was selected as the source of fibroblasts as discussed in section 1.8. This was obtained with full and informed consent (Appendix 2- see patient information leaflet) and ethical approval, from small pieces of urethroplasty grafts which were surplus to requirements. All samples were handled on an anonymous basis under a research tissue bank licence (number 08/H1308/39) under the Human Tissue Authority.

Specimens were cut into 0.5cm^2 pieces and incubated overnight (12-16h) at 4°C in (0.4%) Difco-trypsin plus 0.1% w/v D-glucose in PBS, pH 7.45 (Difco Labs, Michigan, USA). The epidermis was manually parted from the dermis in a Petri dish. The epidermis was discarded and the dermis further minced with scalpel blades in a small volume of 10% DMEM medium (444ml DMEM + GlutaMax™ (Gibco Invitrogen, Paisley, UK) supplemented with 50ml fetal calf serum (Advanced protein products, Brierley Hill, UK) 5ml penicillin (100units/ml), streptomycin(100µg/ml), and 2.5ml fungizone (630ng/ml) (Gibco Invitrogen, Paisley, UK).

The minced dermis was transferred to a Petri dish containing 10ml collagenase A (0.05% in 10%DMEM) and incubated at 37°C in a 5% CO_2 atmosphere overnight. The resulting suspension was centrifuged at 335.4g for 10 minutes and the pellet isolated and resuspended in 10% DMEM. Thereafter a cell count was obtained to guide seeding into T25 flasks, which were seeded with a minimum of 5 000 cells per T25 flask incubated at 37°C in a 5% CO_2 atmosphere. Regular visual inspections were undertaken to observe cell morphology and exclude infection. Media was changed as required or at most every three days and all cells were passaged prior to 80% confluency.

The passage procedure involved three washes in PBS followed by incubation for 5 minutes at 37°C 5%CO_2 atmosphere with 5ml Trypsin/EDTA (Sigma-Aldrich, Dorset, UK) per T75 flask. Thereafter, the suspension was centrifuged at 83.85g for 5 minutes and the cells counted in a haemocytometer. Between 100 000 to 1 000 000 cells were added to each T75 flask, depending on the requirements. Cells between passage 5-9 were utilised in the experiments.

2.3 Material preparation and cell seeding

All material preparation and seeding was undertaken in a sterile laminar flow culture hood reserved specifically for human cell lines. The seven sterile prostheses were cut to $2cm^2$ pieces and placed in 6 well plates. Oral fibroblasts (800 000 per prosthesis), detached by trypsinisation, were seeded on to the cut pieces with a small volume of 10% DMEM. After 30mins, to allow for cell attachment, a further 5ml of 10% DMEM was added and the 6 well plates incubated at 37^oC in a 5%CO2 atmosphere. We also included media free and cell free with media only controls. Each experiment was run in triplicate and repeated three times.

2.4 AlamarBlue®staining

AlamarBlue® (5ml of 10% AlamarBlue in PBS, AbD serotec, Kiddlington, UK) was added to each six well plate one hour after seeding 800 000 cells. This was incubated for 60 minutes at 37^oC in a 5% CO_2 atmosphere and then read at 570nm (manufacturers recommendations) in a colorimetric plate reader (Bio-TEK, NorthStar Scientific LTD, Leeds, UK) to obtain baseline values of colorimetric absorbance. On days 7 and 14, each sample was washed with PBS three times and placed in a new 6 well plate with 5ml AlamarBlue to assess metabolic activity at these time points.

Data analysis involved calculating the % change in absorbance over time relative to day 0 (Baseline) when 800 000 cells were seeded for each of the scaffolds. Differences between scaffolds were statistically tested against the hypotheses (section 1.12) using a two sample T test with equal variance not assumed, n=9 (Significance = $p<0.05$).

2.5 DAPI staining

The DNA stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was utilised to identify the presence and location of fibroblast nuclei via fluorescence imaging. After completion of culture (14 days), cut portions of the tissue engineered prostheses were fixed in 3.7% formalin solution (3.7% formaldehyde in PBS). Thereafter, 0.8ml of 1ng/ml DAPI (Gibco Invitrogen, Paisley, UK) was added to each well (24 well plate) and incubated at 37^oC in a 5% CO_2 atmosphere for 40 minutes. After three washes in PBS, the samples were observed through an Axon ImageXpress™ fluorescent microscope (Molecular Devices limited, Union city, CA) at λ_{ex}385nm/λ_{em}461nm. The samples were imaged through their depth in all quadrants.

2.6 Contraction

Scaffold contraction on days 7 and 14 was calculated relative to day 0 using digital photographs and ImageJ software (NIH, USA). The % contraction at days 7 and 14 was statistically assessed between the 7 scaffolds, against the hypotheses (section 1.12) using a two sample T test with equal variance not assumed, n=9 (Significance = $p<0.05$). Cell mediated contraction was calculated by subtracting the difference between scaffolds with and without cells (controls).

2.7 Tensiometry

Tensiometry was performed on day 14 and all samples remained in media until the time of testing. Samples were cut, measured and then the moist samples were clamped to the tensiometer (BOSE Electroforce test instruments, Minnesota, USA). A small load cell was selected (<22N) as this was found to be the most accurate during sample tuning. All seven scaffolds were tuned separately prior to commencing the experiments. A ramp test was applied at a rate of 0.05mm/s with a maximum displacement of 10mm. The first failure point or plateau was recorded as the load at failure, and the displacement at this point was also recorded. Thereafter the ultimate tensile strength, the ultimate strain and Young's modulus were calculated as described in section 1.10. The ideal biomechanical properties were those of native paravaginal tissue as discussed in section 1.10.2. Statistical differences between native tissue and the 7 scaffolds were assessed, against the hypotheses (section 1.12), using a two sample T test with equal variance not assumed, n=9 (Significance = $p<0.05$).

2.8 Sirius red staining

Total collagen produced by the cells on the scaffolds was assessed using Sirius red staining. At 14 days constructs were washed three times in PBS and fixed in 3.7% formalin. Following a further three washes with PBS the tissue engineered prostheses were stained with 0.8ml Sirius red stain (0.1% Direct Red 80 in saturated picric acid, Sigma-Aldrich, Dorset, UK) to each well of a 24 well plate. After 16 hours, excess stain was washed off with distilled water. The specimens were then pat dried and weighed. For a quantitative analysis, the stain from the tissue constructs was eluted with 2ml 0.2M NaOH: methanol 1:1 to each well in the 24 well plate for

15 minutes. The absorbance was read at 490nm in a plate reader spectrophotometer (Bio-TEK, NorthStar Scientific LTD, Leeds, UK).

Data analysis involved calculating absorbance of stain per gram of dry construct. In addition, the difference in collagen stain absorbance per gram of construct between samples with cells and controls without cells was calculated to give an indication of "new collagen production" by cells on the scaffolds. This is because biological scaffolds already contain a background of collagen. Differences between scaffolds were statistically tested against the hypotheses (section 1.12) using a two sample T test with equal variance not assumed, n=9 (Significance = $p<0.05$).

2.9 Immunofluorescence imaging

The presence and distribution of collagen types I, III, IV and elastin were assessed using Fluorescein labelled (FITC) antibodies. Primary antibodies included goat anti-human collagen I, III, IV, and rabbit anti human alpha elastin antibody (sensitive to both alpha and beta elastin). The respective secondary antibodies included FITC labelled anti goat IgG and anti rabbit IgG (AbD serotec, Oxford, UK). All antibodies were polyclonal.

All samples were fixed in 3.7% formaldehyde, underwent the DAPI staining procedure above and were then washed in PBS thrice. Thereafter 2ml 1% bovine serum albumin was added to each construct in the 24 well plates to reduce non-specific binding. This was washed away with PBS three times after 30 minutes. Then 100µl primary antibody (Diluted 1:50 in PBS) was added, to each sample, and incubated at 37^{o}C in a 5% CO_2 atmosphere for 30 minutes followed by three washes with PBS. Finally 100µl secondary antibody (Diluted 1:50 in PBS) was added and the samples incubated at 37^{o}C in a 5% CO_2 atmosphere for 30 minutes and washed thrice with PBS. The constructs were imaged with an Axon ImageXpress^{TM} fluorescent microscope (Molecular Devices limited, union city, CA). The relative positions of nuclei (DAPI stained) and collagen I,III, IV and elastin were ascertained by switching between DAPI and FITC filters. Excitation and emission wavelengths for FITC were λ_{ex}495nm/λ_{em}515nm. Filters for DAPI have been described above.

Three postgraduate researchers who were presented the images for blind scoring marked each image using a qualitative and quantitative grading scale; - absent= 0, + mild presence=1, ++ good presence =2, +++ abundance =3, (n=9). Both the mean and modal values were presented.

2.10 Scanning electron microscopy

SEM was used to assess new extracellular matrix production on the scaffolds. All samples were fixed in 3.7% formaldehyde. After three washes in PBS, the samples were incubated for 5 minutes with the addition of 2ml 0.1M cacodylate buffer. After removal of the cacodylate, 2ml 2.5% gluteraldehyde in distilled water was added and incubated as above for 30 minutes. The gluteraldehyde was aspirated and again 2ml of cacodylate buffer added to rinse any remaining gluteraldehyde. Thereafter, 500µl of osmium tetraoxide was added and incubated for 2 hours. The osmium tetraoxide was aspirated and 2ml cacodylate buffer added and left for 15 minutes at room temperature. Subsequently the samples were incubated for 15 minutes with 75, 95 and 100% ethanol and finally freeze dried for 16 hours.

A Gold coater (Edwards sputter coater S150B, Crawley, England) was utilised to coat all samples with gold. A Phillips XL-20 scanning electron microscope (Cambridge, UK) was utilised to obtain the SEM images. Three random images were taken for each sample at X100, X800 and X5000 magnification. The samples were then scored for extracellular matrix production by three independent blinded postgraduate researchers. The marking scale included - No ECM, + little ECM, ++ good ECM, +++ abundant ECM. The modal and mean results were calculated.

2.11 Restraint of scaffolds

Restraint was applied to all seven scaffolds using a non-absorbable 5/0 monofilament suture (Ethilon®). The sutures were placed in the four corners of each sample and sutured to a metal grid. The restraint was released on day 14 by cutting the sutures. All the above parameters were assessed with restrained scaffolds and included controls without cells.

2.12 Variable stress rig

The development of a variable stress rig is a continuing work in progress. Thus far we have been able to test an initial rig and an improved version based on the findings of the first rig.

The first rig involved the inverted use of a Scaffdex™ (Scaffdex Oy, Tampere, Finland) cell culture ring. This allowed a constant restraint of the scaffolds. The scaffold was clamped in the ring and placed in a six well plate. Thereafter four ball bearings, weighing 0.261g each, were

placed in the centre of each Scaffdex™ ring on alternating days (Fig 2.11.1). This was done only with the Th PLA scaffold.

The second rig was designed to improve upon the deficiencies of the first rig and utilised the same Scaffdex rings which were cut to reduce the height of the rings. The cut non clamp end was used as a base and the ring clamp side was used as intended by the manufacturer. This allowed the scaffold to rest lower in the 6 well plate (Fig 2.11.2).

2.13 Vitamin C

The addition of vitamin C to the culture media was investigated to examine its impact on cell behaviour, particularly total collagen production. A stable form of vitamin C, Ascorbate-2-phosphate (Asc-2p) (Sigma-Aldrich, Steinheim, Germany) was added to the 10% DMEM media making up concentrations of 0.3, 1 and 3mM. The effect of the different concentrations on cells on Th PLA was investigated using AlamarBlue, DAPI, tensiometry and Sirius red staining.

Chapter 3 Attachment of fibroblasts to scaffolds

3.1 Introduction

Fibroblasts are adherent cells and their survival is dependent upon attachment to a solid surface. Surfaces, such as tissue culture plastic, allow attachment through matrix proteins which adsorb onto the hydrophobic surface. Many researchers have attached fibroblasts to different meshes. Skala *et al*. have shown good attachment of human vaginal fibroblasts to SIS and PD after five hours and five weeks, semiquantitatively, by the use of propidium iodide fluorescence staining [236]. Langer *et al*. reported poor human dermal fibroblast attachment to PPL mesh as assessed by SEM [237]. Kapischke *et al*. noted human foreskin fibroblasts attached well to 3 different PPL meshes as assessed by light and scanning electron microscopy [238]. Two of the meshes were multifilament and the other monofilament and microporous. The authors also showed the presence of collagen I and III using monoclonal antibodies. FACS analysis was performed and showed some increase in the marker Ki67 implying cells were proliferating on the meshes. Kumbar *et al*. showed human skin fibroblast proliferation continued to increase on a PLA/ PLGA scaffold over 28 days via an MTT assay [351]. Ng *et al*. have shown an increase in metabolic activity of human dermal fibroblasts on Alloderm and PLGA/polycaprolactone scaffolds[319]. Confocal and SEM imaging showed a good distribution of cells on the two scaffolds.

In these experiments AlamarBlue® was used to assess the metabolic activity of cells on the seven different scaffolds. This stain has also been used as a surrogate marker of proliferation [428]. It uses an oxidation-reduction (REDOX) indicator, resazurin (blue) which is reduced to resorufin (pink), and can be assessed by fluorescence and colorimetric methods. Importantly the stain is non toxic and can simply be washed away and thus used repeatedly on the same sample without impairing cellular processes [429]. Importantly, the resorufin continually develops and therefore a precise time point is required for assessment.

In addition to the metabolic activity and proliferation of cells, we also assessed the distribution of cells within the scaffolds. For this purpose, fluorescent DAPI staining was used. The axon ImageXpress allows one to assess the whole scaffold and capture sections sliced throughout the scaffolds. The scaffolds and their parameters are shown in table 3.1.1.

Scaffold	Fibre diameter	Pore size mean diameter	Cross linking or modification
AL	A	B	Naturally cross linked only
CD	A	B	Naturally cross linked only
PPL	0.1mm	2.25mm	Woven
PD	A	B	Naturally cross linked only
SF	0.8-11.6 µm	B	Naturally cross linked only
SIS	5µm	0.2-5µm	Naturally cross linked only
Th PLA	2.1µm	5-20µm	Heat annealed

Table 3.1.1. Parameters of the seven scaffolds utilised. A- Data not reported in the literature, however, this material contains natural collagen fibres of 2-300nm diameter forming collagen bundles of approximately 10µm diameter. B-Mean pore size not reported in the literature, however, this material is a natural collagen matrix containing pore sizes up to 10µm.

3.2 AlamarBlue results

AlamarBlue absorbance was read one hour after incubation and was recorded on days 0, 7 and 14. The absorbance of AlamarBlue is plotted below in each figure compared to the absorbance of controls without cells. The results indicate the proliferation and activity of cells over the 14 days of culture.

3.2.1 Alloderm

Fig 3.2.1.1 shows no significant change in absorbance of AlamarBlue stain from AL with cells over 14 days. This implies that cells remain metabolically stable over a 14 day time course when seeded on AL. However the confidence interval at 14 days is wide and is no longer significant when compared to AL without cells. This implies that some scaffolds did well and others less so.

3.2.2 Cadaveric dermis

The metabolic activity of fibroblasts on CD decreased significantly at seven days but thereafter made a significant recovery on day 14 (Fig 3.2.2.1). Metabolic activity was significantly more on the scaffolds with cells than those without throughout the whole 14 days of culture.

3.2.3 Polypropylene

Cells did not survive on PPL. There was a significant reduction in the cellular activity up to day 7, which reached baseline values and continued this way until day 14 (Fig 3.2.3.1).

3.2.4 Porcine dermis

Fig 3.2.4.1. Shows no significant change in absorbance of AlamarBlue stain from PD with cells at 7 (p=0.4) and 14 (p=0.55) days culture in relation to baseline. The difference between PD with and without cells also became non-significant after 7 days culture.

3.2.5 Sheep forestomach

Fig 3.2.5.1. Shows no significant change in absorbance of AlamarBlue stain from SF with cells over 14 days culture. On day 14 the difference between SF with and without cells became significant (p=0.016), which suggests cellular proliferation over time.

3.2.6 Small intestinal submucosa

Metabolic activity as measured by AlamarBlue continued to increase significantly over the 14 days with SIS (Fig 3.2.6.1).

3.2.7 Thermoannealed PLA

Metabolic activity as measured by AlamarBlue continued to increase significantly over the 14 days with Th PLA (Fig 3.2.7.1).

3.3 Comparison of cell metabolic activity on potential scaffold materials

The metabolic activity of cells on the seven different scaffolds was compared by standardising each scaffold to its control (i.e. scaffold without cells). Thereafter, the % change from day 0 when 800 000 cells were seeded on each scaffold, which formed a reference point of 100%, was calculated. Cells on SIS and Th PLA showed the greatest increase in metabolic activity (Fig 3.3.1). PPL showed a decrease in the metabolic activity of cells and the remaining scaffolds remained approximately the same over 14 days.

Table 3.3.1 shows the significant differences between the scaffolds. The third column shows the p value between scaffolds in rank order and the fourth column shows the points at which the p values become significant which allows us to grade the scaffolds (column five). We can see the first two scaffolds did significantly better than the remaining five.

Order	Descending order of materials according to relative activity to controls	P value of difference between material and previous most metabolically active material	Point where P value becomes significant between 1st significant sample and next most significant	Grade
1	SIS			+++
2	Th PLA	0.4		+++
3	SF	<0.001	<0.001	++
4	CD	0.2		++
5	PD	0.65		++
6	AL	0.9		++
7	PPL	0.19	<0.001	+

Table 3.3.1. Materials in rank order of highest metabolic activity achieved by fibroblasts. p values of differences in relative metabolic activity of different materials using two sample T test, equal variance not assumed. n=9 (Significance at P<0.05). Scale: **+++ for best material, ++ for materials if significantly different (p<0.05) from 1st material (using unpaired T-test), + for next material (p<0.05), followed by +/- for next significantly different material (p<0.05).**

3.4 DAPI images

DAPI stains DNA within cells. It was used to assess the distribution of cells within scaffolds. Representative images of DAPI stained scaffolds with and without cells are shown in Fig 3.4.1. Clear differences can be seen between scaffolds with and without cells and between individual scaffolds too. To the naked eye SIS and Th PLA both appear to have significantly more visible nuclei compared to the other scaffolds.

3.5 Discussion

It was hypothesised that all scaffolds would support fibroblast attachment and subsequent performance to some extent. This was based on the available literature at the time of commencing the thesis as discussed in the introduction of this chapter (section 3.1). We found, SIS and Th PLA were significantly better at supporting cell attachment than the other five scaffolds (see Fig 3.4.2 and Table 3.3.1) and PPL was significantly worse than the remaining four scaffolds. Only PPL failed to support fibroblast attachment.

SIS significantly outperformed the other biological scaffolds. The DAPI stained images showed a good distribution of fibroblasts on the SIS (Fig 3.4.1). The remaining biological scaffolds showed variable cell attachment. Cells which were attached to AL, PD and SF did not increase or decrease significantly in metabolic activity over the 14 days of culture (Figs 3.2.1.1, 3.2.4.1 and 3.2.5.1). On day 14, all three materials showed a large variation in their results (large standard error of the mean), which suggests some scaffold samples performed well and others less so. This variation has also been recognised in the literature for biological scaffolds.

AL, CD, SF, SIS and PD are biological scaffolds which have had their cellular components removed. The decellularised matrices are postulated to serve as readymade scaffolds which allow easy attachment of cells to a biological framework. The scaffolds are absorbed over time and new matrix is laid down by local fibroblasts [430]. The reaction of cells to biological matrices is dependent upon two things- the nature/ configuration (including cross-linking) of the implanted matrix and the host immune response to it. The configuration of the matrix may vary depending on the various patented methods of cell removal, sterilisation and subsequent cross linking [431] [432]. In addition, the αGal epitope was highlighted as a potential reason for rejection in humans. Therefore SIS scaffolds have been developed from αGal knockout pigs [433]. Recently it has been shown that the removal of αGal from SIS has not impacted upon its properties [434]. Also of particular importance, biological scaffolds are often cross linked to increase their strength and make them easier to handle. However, this may have unwarranted effects and lead to a chronic inflammatory response resulting in encapsulation [435].

Ng *et al.* found fibroblasts proliferated around the periphery of AL grafts with limited penetration into the matrix over three weeks culture [319]. *In vitro* studies in the mouth have shown phagocytosis occurs in the first few weeks after implantation of AL grafts, followed by remodelling [436]. *In vivo,* Sclafani *et al*. have shown good fibroblast infiltration of CD from biopsies taken from human subjects implanted with it in the skin behind the ear [318], whereas failed CD sling procedures have been shown to lack cellularity and have only peripheral cellular infiltration [321] [214].

Tang *et al.* reported good attachment of 3T3 fibroblasts to PD as assessed by MTT over four days [437]. However, looking at the MTT results, reported by Tang *et al*, there is no significant increase in cell proliferation over the four days of study. Cook *et al*. have shown superiority of porcine SIS compared to PD using synovial and tendon fibroblasts. The SIS scaffold allowed greater retention of viable cells after one week *in vitro*, and *in vivo* showed better replacement with regenerative tissue after six and 12 weeks in a rat abdominal wall model [438]. These *in vitro* findings agree with ours. Armour *et al*. have shown that only 31% of PD grafts showed fibroblast infiltration but the proliferation of human fibroblasts on the PD scaffolds continued to increase over the four weeks of the study [439].

Using AlamarBlue, Englehardt *et al*. have shown an increase in proliferation of urothelial cells seeded on SIS over a 13 day time course thus showing it to be a good scaffold for supporting cells [440]. The reasons behind this ability is yet to be completely elucidated, but it has been suggested that the scaffold configuration and the availability of growth factors in the scaffold may be responsible [333] . The growth factors that have been found to stimulate cell activity are fibroblast growth factor 2, transforming growth factor beta, vascular endothelial growth factor, insulin like growth factor 1 and platelet derived growth factor [441] [434]. In addition to this, SIS has been shown to have inherent antimicrobial activity [442].

The ability of SF has not been compared in the literature to other materials. However, Irvine *et al*. have shown that SF led to greater blood vessel formation than an SIS matrix on an untreated wound in a pig wound model [443]. We seeded cells on the luminal surface of SF which only showed an increase in metabolic activity on day 14. Recently, the abluminal surface of SF has been shown to allow better attachment of fibroblasts as assessed by manual counting of DAPI stained scaffolds [339].

PPL and Th PLA are both synthetic scaffolds. PPL is made of knitted and interlocked filaments to create a macroporous non absorbable mesh. Fibroblasts did not attach well to the PPL except at the interlocking junctions where smaller pores existed. The cells can be seen, by DAPI, to congregate at the interlocked junctions but are not able to extend from one junction to the next (Fig 3.4.1). This is similar to the findings of Skala *et al*. who used immunofluorescence microscopy and showed that fibroblasts could attach to mesh nodes but not bridge the large pores [323]. In contrast Kapischke *et al*. showed fibroblasts were able to attach to PPL mesh at four to six weeks but the mesh used had small pores (0.26mm) [238]. It has been suggested that cells may not be able bridge large pores, whereas with small pores which allow cell to cell communication cells may be able to proliferate better [444].

Electrospun random fibres of PLA allowed good attachment of fibroblasts, as viewed by DAPI (Fig 3.4.1), and cells showed good metabolic activity on these scaffolds (Fig 3.3.1). In the literature a flow perfusion bioreactor was reported that led to better organisation and more homogeneity of fibroblasts on PLA, in addition to increasing fibroblast numbers [445]. This also led to quicker *in vitro* maturation by simulating an *in vivo* environment. Other methods of increasing cell adhesion and proliferation are to modify the PLA surface with a coating such as gelatin [446] or to form co-polymers with other materials [447]. These unfortunately affect the mechanical properties and also the extent of collagen and elastin deposition. The reasons for the success of PLA have been postulated to be the fibre diameter (2.1$\pm$ 1.2µm), pore size (5-10 µm) and arrangement (random). These properties create a matrix close enough to native tissue (Table 3.1.1) to encourage fibroblasts to attach, proliferate and produce new ECM.

It has been suggested that it may be the hydrophobicity of PLA which encourages fibroblast attachment. However, it has also been shown that when collagen was blended with PLA it made the scaffolds more hydrophilic and hydroxyapatite made them more hydrophobic. Scaffolds combined with collagen and hydroxyapatite with PLA were shown to have the most viable cells [448]. Another study added polyethylene glycol to PLA to increase hydrophillicity. The authors found an 80:20 ratio allowed for the best cell-matrix interaction [449]. One must, however, bear in mind that this will change other properties of the co-scaffold [450]. Heat annealing slows hydrolytic degradation rate and increases hydrophobicity [451]. This may explain why fibroblasts attached well to the Th PLA.

These are the first results, to my knowledge, that compare the ability of these seven scaffolds to support fibroblasts in this way. There is no accepted standard measurement in the literature to measure cell attachment and activity. We have shown two scaffolds to be significantly superior to the other scaffolds for the attachment of fibroblasts. Later in the thesis, the effects of adding vitamin C to the culture media and of culturing cells on scaffolds under fixed or dynamic tension are examined for cells grown on the Th PLA scaffolds.

Other scaffolds which have shown recent promise in the literature include human recombinant tropoelastin which is electrospun to resemble a dermal matrix [452]. The authors reported good cellular attachment and ECM production but the biomechanical properties were those that resembled skin and not paravaginal tissue. Recently, the use of gelatin as a co-polymer with other scaffold materials has also shown benefit to fibroblast attachment [453] [454] [455]. The use of patterned electrospun fibres has also shown some degree of benefit [456] [457].

Fibroblasts which integrate into scaffolds have been shown to display a different morphology to those in monolayers due to cell-matrix entanglement [458]. It has been shown fibroblasts may migrate in a 3D matrix via a chemoattractant gradient, most notably platelet derived growth factor [459] [460] [461]. The mechanical loading of scaffolds has been shown to influence whether cells acquire a proliferative or resting phenotype [462]. Therefore, the measurement of bioactive substances produced by fibroblasts on the scaffolds may also improve our understanding of why fibroblasts prefer one scaffold over another. Moreover, it will help us understand what cellular processes occur at various time points and this may lead to an avenue for modulation. In addition to the initial attachment and growth of cells on the scaffolds, the biomechanical properties, contraction and production of ECM are also important and these are considered in the following chapters. The importance of cell attachment to tissue engineered scaffolds for use in SUI/POP is considered further in the main discussion in chapter 10.

Chapter 4 The mechanical properties of scaffolds seeded with fibroblasts

4.1 Introduction

The biomechanical properties of scaffolds are hypothesised to affect their ability to integrate into native tissue (section 1.10). Although in our review of this area, we found no absolute correlation between prosthesis success/ erosion rate and the UTS or YM, it must be noted that the description of the biomechanics of these properties is limited in the literature (Section 1.10.5). In addition, it seems reasonable to propose that the biocompatibility of prostheses and the ability of meshes to integrate with native tissue will play a significant part in their success. Accordingly, the relative contribution of the biomechanics of a prosthesis to its success is not known at the present time.

As discussed in section 1.10, there are no standardised uniaxial testing protocols. We used a protocol described by our group previously [463]. The calculation of stress vs. strain data takes account of the tissue volume being tested and thus is comparable between different studies. The effect of small variations in testing have been shown to be negligible as discussed in 1.10. All scaffolds were tested immediately after removal from media for standardisation.

We found no reports in the literature of the assessment of the biomechanical properties of the seven scaffolds we studied after culture with fibroblasts. The effect fibroblasts may have on scaffolds has been investigated with other materials. Liao *et al*. have demonstrated a decrease in the YM of polyethylene glycol diacrylate after culture with vocal fold fibroblasts for 30 days [464]. These authors revealed that collagen production was largely unaffected but elastin production increased with decreasing YM. Selim *et al*. showed a decrease in the UTS and increase in the YM of PLGA:PLA (85:15) scaffolds cultured with cells [463]. The UTS and YM of collagen gels was shown to decrease after 6 days culture with human and mouse fibroblasts [465]. The converse has also been reported by Ouasti *et al*. who reported that a higher YM led to increased cell spreading of fibroblasts, however, the scaffolds with higher YM had increased amounts of hyaluronic acid which the authors claimed would not have ordinarily led to such increases in YM [466].

As there are no reports in the literature of the change in the mechanical properties of the seven scaffolds after the addition of cells, our null hypothesis is that “the addition of cells will not significantly affect the mechanical properties of the seven scaffolds”. The mechanical properties investigated were the UTS, UT strain and YM as discussed in section 1.10. The ranges for healthy native tissue discussed in section 1.10.2 will form the target range for

comparison between materials. Based on the current literature, it is hypothesised that, the mechanical properties of PPL, PD, SF and Th PLA will be closest to native tissue.

4.2 Tensiometry results

We assessed the UTS, UT strain and YM of all seven scaffolds with and without cells. These were compared to the range for native healthy paravaginal tissue.

4.2.1 Alloderm

We found no significant difference in the mechanical properties of AL with and without cells. The UTS is the only parameter for which AL is in the correct range (Fig 4.3.1). The UT strain (fig 4.3.2) is higher and the YM (Fig 4.3.3) lower than required for native tissue. This makes AL more accepting of stretch than required.

4.2.2 Cadaveric dermis

The mechanical properties of CD were not significantly affected by the addition of fibroblasts. The UTS and UT stain are higher than native tissue and YM is lower thus making the scaffold stronger but stretchier than native tissue (Figs 4.3.1, 4.3.2 and 4.3.3).

4.2.3 Polypropylene

PPL remained unchanged by the addition of cells and culture for 14 days. The YM of PPL is slightly less than native tissue, (Fig 4.3.3) whereas the UTS is three fold higher (Fig 4.3.1). The UT strain is within the range for native tissue (Fig 4.3.2).

4.2.4 Porcine dermis

PD showed no change in its biomechanical properties after the addition of cells for 14 days. Although the UTS and UT strain are within the range of healthy tissue, the YM is approximately

40% of native tissue (Figs 4.3.1, 4.3.2 and 4.3.3). This suggests it accepts stretch more readily than native tissue.

4.2.5 Sheep forestomach

SF was the only material to show a significant difference (p=0.02) between the UT strain of scaffolds cultured with and without cells after 14 days (Fig 4.3.2). This is in the range of native tissue. SF has a UTS six fold higher, and a YM that is at the upper limit of non-prolapsed tissue (Fig 4.3.1 and 4.3.3).

4.2.6 Small intestinal submucosa

SIS has a UTS and UT strain much higher than healthy tissue (Fig 4.3.1 and 4.3.2). The YM was found to be at the lower end of the native tissue range (Fig 4.3.3). The changes in the mechanical properties with and without cells were not significant.

4.2.7 Thermoannealed PLA

Th PLA was within the range of native tissue for all three parameters and was not significantly affected by culturing with cells for 14 days (Figs 4.3.1, 4.3.2 and 4.3.3).

4.3 Comparison of materials

Materials were ranked in order of those closest to native tissue (Table 4.3.1). The statistical difference was calculated between each material and native tissue. The materials were then graded according to the statistical difference between them.

In terms of UTS; Th PLA, AL and PD are not statistically different from native tissue (Table 4.3.1). When looking at UT strain; PD, Th PLA, PPL and SF are statistically within the range for healthy tissue (Table 4.3.2). Only SF and Th PLA have the same YM as native tissue (Table 4.3.3). Therefore the only material to be in the native tissue range for UTS, UT strain and YM is Th PLA.

Order	Descending order of materials according to UTS closest to native tissue	p value of difference between material and native tissue	Point where p value becomes significant between 1st significant sample and next most significant	Grade
1	Th PLA	0.56		+++
2	AL	0.43		+++
3	PD	0.41		+++
4	CD	0.003	0.001	++
5	PPL	0.002		++
6	SIS	0.002		++
7	SF	0.0005	0.007	+

Table 4.3.1. Comparison of UTS. Materials are shown in rank order of being closest to native tissue. Third column shows p values of differences in UTS of materials with cells against native tissue using one sample T test. Fourth column shows significant differences between scaffolds using a two sample T test, equal variance not assumed. n=9 (Significance at P<0.05). Scale in fifth column: +++ for best material, ++ for materials if significantly different (p<0.05) from 1st material (using unpaired T-test), + for next material (p<0.05), followed by +/- for next significantly different material (p<0.05).

Order	Descending order of materials according to UT strain closest to native tissue	p value of difference between material and native tissue	Point where p value becomes significant between 1st significant sample and next most significant	Grade
1	PD	0.96		+++
2	Th PLA	0.96		+++
3	PPL	0.8		+++
4	SF	0.5		+++
5	AL	0.046	0.002	++
6	SIS	0.02		++
7	CD	0.01		++

Table 4.3.2. Comparison of UT strain. Materials are shown in rank order of being closest to native tissue. Third column shows p values of differences in UT strain of materials with cells against native tissue using one sample T test. Fourth column shows significant differences between scaffolds using a two sample T test, equal variance not assumed. n=9 (Significance at P<0.05). Scale in fifth column: +++ for best material, ++ for materials if significantly different (p<0.05) from 1st material (using unpaired T-test), + for next material (p<0.05), followed by +/- for next significantly different material (p<0.05).

Order	Descending order of materials according to YM closest to native tissue	p value of difference between material and native tissue	Point where p value becomes significant between 1st significant sample and next most significant	Grade
1	SF	0.19		+++
2	Th PLA	0.2		+++
3	SIS	0.01	0.01	++
4	PPL	0.005		++
5	CD	<0.0001		++
6	PD	<0.0001	0.03	+
7	AL	<0.0001		+

Table 4.3.3 Comparison of YM. Materials are shown in rank order of being closest to native tissue. Third column shows p values of differences in YM of materials with cells against native tissue using one sample T test. Fourth column shows significant differences between scaffolds using a two sample T test, equal variance not assumed. n=9 (Significance at P<0.05). Scale in fifth column: +++ for best material, ++ for materials if significantly different (p<0.05) from 1st material (using unpaired T-test), + for next material p<0.05, followed by +/- for next significantly different material.

4.4 Discussion

These findings show that culturing the seven materials with and without cells did not affect the mechanical properties significantly except for the UT strain of SF. The findings in the literature, discussed in the introduction above, do not however agree with our findings. This may be due to the different materials used; for instance the collagen gels used by Saddiq *et al*. [465] would be more prone to the effects of fibroblast digestion and remodelling. This may also be true for the PLGA:PLA mixed scaffold used by Selim *et al*.[463], which has been shown to degrade faster than pure PLA [367]. Clearly, besides the speed of degradation, the length of time in culture may also lead to different changes.

Once fibroblasts have attached to scaffold fibres they may begin to spread out via chemical mediators either from the cells or from the surrounding ECM. The physical properties of the scaffold have been shown to affect this outgrowth of cells [467]. Fibroblasts exhibit pseudopodia which aid distant cell attachment and movement [468]. Thereafter, the fibroblasts either produce matrix metalloproteinase to aid the breakdown of the scaffold or produce ECM to reinforce the scaffold. The scaffold properties have been shown to dictate this behaviour [469]. Fibroblasts may also differentiate to myofibroblasts, which have contractile properties [470]. At high cell counts cell-cell contact inhibition occurs.

The difference in the mechanical properties will be based on these changes. One may expect, the production of new ECM including collagen and elastin by the fibroblasts to lead to a

significant change in the mechanical properties, however, a lack of this may be due to a lack of ECM production or immature ECM which adds no significant strength to the scaffold. In addition, the scaffolds tested are stronger than those reported in the literature and therefore changes to their properties will not be as apparent.

The scaffold closest to native tissue in UTS, UT strain and YM was Th PLA. One must note, that nine months implantation *in vivo* led to decreases in UTS and YM in the majority of scaffolds (section 1.10.4), possibly due to scaffold degradation by macrophages [283] [284] . Therefore, should we be aiming to create prostheses that will lead to the same mechanical properties as native tissue after implantation?

It must be borne in mind that none of the scaffolds described in section 1.10.4 were cellular. Therefore, the scaffolds only had the intrinsic ability to undergo degradation or stimulate local fibroblasts to produce new ECM. We already know that local fibroblasts in patients with SUI/POP may produce deficient matrix [471]. Therefore could the implantation of laboratory expanded autologous buccal fibroblasts lead to new and stronger ECM production which maintains itself over time? If new matrix production leads to maintenance of the biomechanical properties then it is totally reasonable to aim, as we did, to produce prostheses with the mechanical properties of healthy native tissue. This will clearly need further investigation in animal models.

In conclusion, the knowledge gained from these studies allows us to design a prosthesis and aim to achieve the ideal mechanical properties of native tissue but this remains a new area where there are several basic question yet to be answered. These are further examined in the main discussion in chapter 10.

Chapter 5 Contraction of scaffolds seeded with fibroblasts

5.1 Introduction

Scaffold contraction is often a nuisance in tissue engineering applications [472]. However, with this application, a degree of contraction would be a welcomed feature. Approximately 14-25% prosthetic contraction is noted with current prostheses such as PPL in a rat abdominal wall model [370] [304]. There is no consensus on how much contraction is appropriate for a pelvic organ prosthesis. Excessive contraction leads to induration of tissues and pain and a total lack of contraction may lead to loose tissues thus increasing the risk of recurrence of SUI/POP [473].

Some authors have suggested that with current PPL meshes, 15% contraction per year may occur continuing up to 85% [474]. However Dietz *et al*. have shown no contraction of mesh occurred from three months to 18 months in 40 women, concluding that the initial mesh contraction was probably part of integration and remodelling and was non progressive [475]. This was the only study that followed up patients over more than two time points after implantation. Others have shown a reduction in mesh size compared to the time of implantation [476] [477]. More recently, Svabik *et al*. have shown that in addition to contraction some folding of mesh also occurs which may give the appearance of contraction [478]. The authors assessed mesh size before surgery, then four days and thereafter four months after surgery. Initial reduction in mesh size at four days was thought to be due to folding of mesh whereas later reduction in size was explained as mesh contraction.

In vitro, however, the contraction of meshes is well recognised. This is in part due to a lack of attachment or restraint of the scaffolds which is not the case *in vivo.* Mesh contraction *in vitro* is important in giving us an idea of the size of mesh to commence with. Clearly, contraction which occurs post implantation requires *in vivo* study. However, it is hypothesised that investigating cell mediated contraction *in vitro* may help predict if *in vivo* contraction may occur.

Scaffold contraction was assessed with serial digital photographs. I was hypothesised that in the tissue engineered prostheses the extent of scaffold and cell based contraction will relate to the stiffness (Young's modulus) of the scaffolds.

5.2 Scaffold contraction results

Change in % contraction from baseline over 14 days in scaffolds with and without cells cultured in media was assessed. Dry scaffolds did not undergo any contraction (data not presented).

5.2.1 Alloderm

AL underwent approximately 3% contraction over 14 days (Fig 5.2.1). This was not significantly different from day 0. Cells did not affect this contraction. AL has a low YM and also did not undergo significant contraction (Fig 5.3.2).

5.2.2 Cadaveric dermis

CD underwent significant contraction (16%) in the first 7 days. Thereafter further contraction was not significant (Fig 5.2.2). There was a significant increase in the contraction of scaffolds with cells. This is related to a high YM of 18MPa. This suggests cell mediated contraction with CD even in the presence of high YM.

5.2.3 Polypropylene

PPL did not undergo any significant contraction with and without cells (Fig 5.2.3). It has a relatively high YM which may explain its low contraction (Fig 5.3.2).

5.2.4 Porcine dermis

PD did not show much contraction over 14 days (Fig 5.2.4). The only significant contraction of 4% occurred between days zero to seven in scaffolds with cells. PD has a low YM and there was no difference between scaffolds with and without cells.

5.2.5 Sheep forestomach

SF showed a very high YM (Fig 5.3.2) and did not show any significant contraction over 14 days (Fig 5.2.5).

5.2.6 Small intestinal submucosa

The majority of scaffold contraction occurred within seven days with SIS (Fig 5.2.6). There was no significant difference between scaffolds with and without cells. This implies the contraction was not cell mediated. The YM of SIS is high (Fig 5.3.2).

5.2.7 Thermoannealed PLA

Similar to SIS and CD, the majority of contraction with Th PLA occurred within the first seven days of culture with and without cells (Fig 5.2.7). Again contraction was scaffold based. Scaffold YM was relatively low.

5.3 Comparison of materials

Fig 5.3.1 shows the % contraction of each scaffold with cells. CD, SIS and Th PLA contracted to approximately 15%, PD contracted to 7%, and, AL, PPL and SF showed no significant contraction. As there is no agreed definition of the amount of contraction that may be beneficial we did not score scaffolds with respect to this but calculated the significance of the difference between scaffolds with and without cells (Table 5.3.1). This shows the significance of cell mediated contraction. We also found no significant correlation between scaffold YM and scaffold contraction; Pearson's correlation = -0.23, p=0.62 (Fig 5.3.2).

Scaffold	% contraction over 14 days of scaffolds without cells	% contraction over 14 days of scaffolds with cells	p value of the difference between scaffolds with and without cells
PPL	1.53	1.18	078
SF	4.24	2.26	0.46
AL	2	3.4	0.63
PD	4.4	7.1	0.29
Th PLA	10.9	14.1	0.51
SIS	14	15.4	0.52
CD	7.7	17.6	0.017

Table 5.3.1. The % contraction of scaffolds with and without cells, significance = $p<0.05$.

5.4 Discussion

Scaffold contraction was shown to occur independently of YM and thus the null hypothesis was rejected. The extent of scaffold contraction was a predictive feature of the materials. Most contraction occurred within the first seven days. With the exception of CD, scaffold contraction was not shown to be cell mediated and occurred with scaffolds kept in culture medium over 14 days.

AL and CD are derived from the same source. However, AL undergoes a different patented decellularisation process [479]. This results in a more cross linked scaffold which has also shown to be resistant to contraction when cultured with human dermal fibroblasts [319]. CD however, did undergo significant cell mediated contraction. In *in vivo* models, the use of AL has been shown to lead to reduced contraction in skin [480], abdominal wall [481], eyelid [482], and diaphragmatic models [483]. Contrastingly, PD and SIS have shown no benefit in reducing skin wound contraction when applied to mouse skin models [484]. Therefore, it may be the ability of the scaffold to resist softening by media and cells, which is independent of their YM that prevents them from contracting. Biological scaffolds are postulated to contract on exposure to media due to release of cross linked bonds. A biological material *in vivo* is under constant strain and lacks a toe in region when performing uniaxial testing. The amount of non-biological cross linking which occurs during preparation will dictate contraction once media is added.

Harley *et al*. have discussed the various methods which may be used to measure the contractile force generated by fibroblasts in contracting scaffolds [485]. The mechanisms by which fibroblasts cause contraction is twofold; firstly fibroblasts can slowly contract free floating gels by tractional remodelling [486], secondly by assuming the form of myofibroblasts; contraction may be achieved by retraction of pseudopodia and collapse of actin filament bundles. Tissue strain is proposed to induce this change to the myofibroblast phenotype and once this strain is removed fibroblasts will revert to their normal phenotype [487]. Fibroblasts have been proposed to utilise microtubule dependant dendritic extensions to provide a mechanical structure to enable contraction as well as actin-myosin activity when under greater stress [488]. Grinnell has shown that cell based contraction may occur secondary to mechanical loading as a response to the tension applied to the fibroblasts [489]. The four ply SIS has been shown to contract to 29% after implantation as a jejunal segment in rats after 8 weeks, in contrast to the one ply which contracted to 6% [490]. This may therefore explain why we observed minimal cell mediated contraction and thus fixed scaffolds may induce

fibroblasts to increase this contraction. In addition to this, it has been shown that Platelet derived growth factor and lysophosphotidic acid may induce matrix contraction [491].

There are no studies investigating contraction of SF scaffolds either *in vitro* or *in vivo* and therefore our findings are important in clarifying this. PLA:PLGA has been shown to contract up to 50% within the first five days irrespective of whether cells were attached, followed by a further 10% by day 19 [463]. We used thermoannealed PLA as this has been shown in our laboratory to reduce scaffold contraction (unpublished work). PPL mesh has been shown to contract by 31% compared to 25% if restrained [492], and increased contraction has been linked to reduced tissue ingrowth [444]. The difference in contraction between PPL mesh and Th PLA mesh may be due to the electrospinning process. This leads to stretching of PLA fibres as they are sprayed on to the collecting drum. Upon exposure to media this leads to softening of stretched intercommunications which allow contraction. PPL is not produced in a stretched fashion and therefore this phenomenon is absent with PPL.

As there is no definition of the desired amount of contraction, we were unable to grade the scaffolds accordingly. However, knowledge of the amount of *in vitro* contraction is important in calculating the starting scaffold size. As the rate of scaffold contraction had slowed down between days seven to 14, it would not be expected for further significant contraction to occur beyond this point. One may aim to investigate contraction over a longer time course but it is not envisaged that pre implantation culture will last longer than this. Finally as discussed above *in vitro* contraction may give an indication of *in vivo* contraction.

Further consideration of how this knowledge relates to a tissue engineered scaffold is discussed in the main discussion in chapter 10.

Chapter 6 Extracellular matrix production by fibroblasts on scaffolds

6.1 Introduction

A suitable scaffold should not only encourage the attachment and proliferation of fibroblasts but also the production of new ECM throughout the matrix. Ng *et al*. showed collagen I, III and fibronectins only in the periphery of Alloderm matrix seeded with dermal fibroblasts but a more homogenous distribution of ECM was seen with PLGA-PCL scaffolds [319]. Kapishke *et al.* showed high expression of collagen I on PPL meshes but low expression of collagen III as assessed by immunostaining [238].

The production of ECM proteins on the scaffolds *in vitro* is clearly a reflection of cell survival and proliferation on the scaffolds. This forms the basis for continued production of ECM *in vivo*. The importance of each ECM component is discussed in section 1.11. It is not the aim to create a completely formed connective tissue pre-implantation, but rather to show that the capability to produce ECM exists in the tissue engineered implants.

It was hypothesised that the biological scaffolds will facilitate the production of collagen and ECM significantly more than the synthetic scaffolds. To test this hypothesis we assessed total collagen production using Sirius red staining, and collagen I, III, IV and elastin production using immunostaining and also assessed total scaffold ECM coverage using scanning electron microscopy (SEM).

6.2 Total collagen production results

New collagen production by cells on scaffolds was assessed by standardising with control scaffolds without cells. The absorbance of Sirius red stain per gram is presented in Fig 6.2.1. SF, SIS, Th PLA and AL were significantly better than the remaining scaffolds for collagen produced by fibroblasts (Table 6.2.1).

Order	Descending order of materials according to most Sirius red stain per gram of scaffold	P value of difference between scaffold and next ranked scaffold	Point where P value becomes significant between 1st significant sample and next most significant	Grade
1	Th PLA			+++
2	SIS	0.21		+++
3	SF	0.52		+++
4	AL	0.57		+++
5	CD	0.97	0.02	++
6	PPL	0.08		++
7	PD	0.83		++

Table 6.2.1. Comparison of new collagen production. Materials are shown in rank order of most Sirius red stain per gram. Third column shows p values of differences in stain per gram of materials in rank order and fourth column shows significant differences between scaffolds using a two sample T test, equal variance not assumed, n=9 (Significance at $p<0.05$). Scale in fifth column: +++ for best material, ++ for materials if significantly different ($p<0.05$) from 1st material (using unpaired T-test), + for next material ($p<0.05$), followed by +/- for next significantly different material.

6.3 Immunostaining for collagen I, III, IV and elastin results

We assessed for the presence of collagen I, III, IV and elastin on all the scaffolds with and without cells using immunostaining. Cell nuclei within scaffolds were initially stained with DAPI followed by a fluorescent stain for the particular ECM protein. Some positive images for Th PLA and SIS with cells are shown in Fig 6.3.1 and Fig 6.3.2. Three postgraduate researchers who were presented the images for blind scoring marked each image using the scale below (n=9). In Table 6.3.1 the modal value and mean of the three researcher's scores are presented. The mean $\pm$ SEM is also shown in Fig 6.3.3 for collagen I, Fig 6.3.4 for collagen III and Fig 6.3.5 for Elastin.

The results for collagen IV are not presented as there was no evidence of any production of this in culture. With AL and CD there was a small amount present intrinsically in these scaffold but no evidence of any collagen IV being produced by the cells during the period of culture.

	Collagen I		Collagen III		Collagen IV		Elastin	
Material	Mode	Mean	Mode	Mean	Mode	Mean	Mode	Mean
AL without cells	++	1.56	+	0.56	-	0.22	-	0.22
AL with cells	++	1.67	+	0.56	-	0.11	+	0.67
CD without cells	++	1.56	+	0.67	-	0.22	-	0.22
CD with cells	++	1.56	+	0.75	-	0.11	-	0.33
PPL without cells	-	0	-	0	-	0	-	0
PPL with cells	-	0	-	0	-	0	-	0
PD without cells	-	1.56	-	0	-	0	-	0
PD with cells	-	1.56	-	0	-	0	-	0
SF without cells	+	0.44	-		-	0	+	0.67
SF with cells	+	0.67	-		-	0	-	0.22
SIS without cells	+	0.67	-	0.44	-	0	+	1
SIS with cells	++	1.67	+	0.89	-	0	++	1.67
Th PLA without cells	-	0	-	0	-	0	+	0.67
Th PLA with cells	++	1.78	+	1	-	0	++	1.56

Table 6.3.1. Summary of immunostaining results. Modal and mean values are presented as reported by three blinded researchers (n=9). Qualitative and quantitative grading scale; - absent= 0, + mild presence=1, ++ good presence =2, +++ abundance =3.

6.4 Use of SEM to assess extent of ECM production

The assessment of ECM by immunostaining utilised three postgraduate researchers who assessed the presence of ECM on the seven scaffolds. These are presented as the modal and mean values in Table 6.4.1 and graphically in Fig 6.4.1. Representative images are shown in Fig 6.4.2.

Material	Mode	Mean
AL without cells	+++	2.78
AL with cells	+++	2.44
CD without cells	+++	2.78
CD with cells	+++	2.56
PPL without cells	-	0
PPL with cells	-	0.11
PD without cells	+++	2.78
PD with cells	+++	2.78
SF without cells	++	1.78
SF with cells	++	1.56
SIS without cells	+	1.22
SIS with cells	+	1.33
Th PLA without cells	-	0.11
Th PLA with cells	+	1.22

Table 6.4.1. Summary of SEM results. Modal and mean values are presented as reported by three blinded researchers (n=9). Qualitative and quantitative grading scale; - ECM absent= 0, + mild presence=1, ++ good presence =2, +++ abundance =3.

6.5 Discussion

The results show that most collagen was produced by fibroblasts on the Th PLA, SIS and SF scaffolds (Fig 6.2.1). The immunostaining and SEM images further clarified these results.

AL and CD intrinsically showed a mild to good level of collagen I and elastin present in the absence of cells which did not change with the addition of cells (Fig 6.3.3 and 6.3.5). Collagen III was only mildly present on these scaffolds with and without cells (Fig 6.3.4). Similarly SEM scores also did not indicate any increase with the addition of cells (Fig 6.4.1). As these natural collagenous human skin derived scaffolds are rich in ECM proteins such as collagen and elastin, the ability to detect any new collagen and elastin production may be difficult against this background - a limitation of this assessment methodology. Similarly, PPL and PD showed no change in any of the investigated ECM products with the addition of cells. This suggests that cells did not produce any new ECM products on these two scaffolds.

SF, SIS and Th PLA are seen to be good scaffolds which enabled cells to produce collagen I and III (Fig 6.3.3 and 6.3.4). More elastin was seen to be produced by cells on Th PLA and SIS than on SF (Fig 6.3.5). Only Th PLA seems to show a large difference between scaffolds with and without cells when assessing ECM production as seen by SEM (Fig 6.4.1). This is probably because as Th PLA is a synthetic scaffold without any intrinsic ECM the appearance of ECM on this scaffold is more easily detectable as can be seen in the representative images in Fig 6.4.2.

A further limitation of this semi quantitative assessment is that the scale is limited by human interpretation and the ability to detect visual differences. Therefore only a four point scale was selected. Even with this there were differences in reported scores by three independent assessors. We have presented the modal and mean values. Neither of these can be used for statistical comparison but can be used to give an indication of ECM protein production.

We assessed the presence of collagen I, III, IV and elastin on DAPI stained scaffolds. The presence of these ECM products can be seen to be associated with the presence of cells (Fig 6.3.1). Collagen IV was only seen in AL and CD scaffolds as it is intrinsic to their structure. It was not produced by fibroblasts on any of the scaffolds over the period of 14 days culture. This is because it is principally seen in the basal lamina which is absent in connective tissue [493]. It has also been shown that an air liquid interface and keratinocytes may be required for epithelial differentiation and production of a basal lamina [494]. Pena *et al.* have shown stratification and basement membrane formation, via immunostaining for collagen IV and laminin 5, using both oral fibroblasts and keratinocytes on an autologous fibrin scaffold [495].

The basal lamina took 21 days to form. Other studies have also shown the presence of collagen IV only after three weeks *in vitro* [496].

In section 1.11.7, we report a review of the literature regarding the deficits in collagen and elastin in the tissue of women with SUI/POP. There is only a small amount of relevant published literature in this area-some studies report collagen I, III and elastin to be reduced and others state that these are unchanged in women with SUI/POP (Table 1.11.7.1). Therefore the production of these by fibroblasts is clearly important. We have shown that fibroblasts may produce these proteins on the SIS and Th PLA scaffolds *in vitro* but whether this continues *in vivo* clearly requires an *in vivo* study. Rouabhia and Allaire have shown that a tissue engineered oral mucosa equivalent consisting of a collagen scaffold, oral fibroblasts and keratinocytes survived well when implanted in to the dorsal skin of a rat model [497]. The authors showed histological evidence of a well organised epithelium at 60 days, and immunological evidence of angiogenesis. Interestingly there were no hair follicles on the transplanted oral mucosa, implying that the native skin fibroblasts/ keratinocytes had not invaded into the transplanted tissue.

The reasons why some scaffolds were better than others at encouraging ECM production may be due to the ability of fibroblasts to attach to them. Table 3.3.1 shows that the scaffolds on which most cells attached and were metabolically active were SIS and Th PLA followed by SF. These are the scaffolds upon which most ECM was produced. It has, however also been shown that scaffold geometry can affect the ability of fibroblasts to produce ECM [489]. If the ECM is seen as being different and fibroblasts are capable of attaching to the matrix then the fibroblasts will secrete a new matrix to modify their own environment [498]. This in turn could be responsible for the increased metabolic activity of cells on those scaffolds.

Type I collagen plays a critical role in tissue strength. Therefore the production of significant amounts of type I collagen should increase the overall scaffold strength. In Fig 4.3.1 we see that the UTS of SIS decreased by the addition of cells and that of Th PLA did not change significantly. This may be due to the production of immature collagen which after two weeks is not organised sufficiently to increase the UTS and in addition the SIS scaffold may have undergone some degradation which led to a decrease in UTS.

Similarly, elastin is thought to decrease the YM of tissues and make them more elastic. The three tissues which showed increased elastin production were AL, SIS and Th PLA (Fig 6.3.5). The YM of AL and Th PLA did not change significantly (Fig 4.3.3) but that of SIS decreased making it more elastic. However, this may be the effect of scaffold degradation as the UTS decreased as well.

More recently, using media obtained from Mesenchymal stem cells, Jeon *et al*. showed that this media could increase fibroblast survival and production of collagen, elastin and fibronectin [499]. Other methods of increasing ECM production by cells may also be employed. Eming *et al*. have reported the use of high PDGF-A expressing keratinocytes to produce increased collagen I and IV [500]. This also led to reduced contraction of scaffolds. Sarukawa *et al*. have shown increased fibroblast proliferation and ECM production on PLA scaffolds coated with chitosan [501]. Ascorbate, fibronectins, velvet antler extract and angiotensin II have also been shown to stimulate collagen production by fibroblasts in cell culture [502] [503] [504] [505]. In addition to this, mechanical uniaxial strain has been shown to increase collagen production by fibroblasts [506].

Mariotti has reported the use of 1nM estradiol with gingival fibroblasts, to reduce collagen I production on plastic and collagen IV matrices, and non-collagen protein production to decrease on plastic and collagen I matrices [507]. This shows that fibroblasts behave differently on different matrices; which is in keeping with our findings.

In summary, this data suggests that the presence of existing ECM and auto fluorescence of biological scaffolds makes the delineation of new ECM produced by added fibroblasts very difficult to detect in these scaffolds. The future use of western blotting may help with a more quantitative assessment of ECM production [508]. As expected in synthetic scaffolds it is easy to detect new ECM. The results did show however the presence of new ECM orientated adjacent to fibroblasts in the synthetic PLA scaffold which is what we wished to establish in developing a tissue engineered prosthesis.

Chapter 7 The effect of restraint on the production of tissue engineered prostheses

7.1 Introduction

Restraint has been used to induce tissue growth and remodelling of tissue engineered tissue [509]. The use of bioreactors has been recognised as an efficient way to apply mechanical stress to scaffolds cultured with cells. Stress may be static or dynamic and Kanda and Matsuda have shown that dynamic stress led to smooth muscle cells with increased contractile apparatus, whereas static stress led to an increase in cellular synthetic apparatus within the smooth muscle cells [510]. Bioreactors have been used mostly to apply dynamic stress in the tissue engineering of cardiovascular tissue where a pulsatile flow is often used to mimic arterial flow [511].

Static load has also been shown to increase the tensile strength of tissue engineered constructs with fibroblasts by inducing cellular and ECM alignment towards the axis of stimulation [512]. Using 10% strain via a clamp within a bioreactor, Gauvin *et al*. showed increased ECM production by dermal fibroblasts on a self assembled ECM matrix [508]. Elastin was shown to be increased by static strain and cells and ECM were seen to be aligned in parallel to the axis of stress. This led to an increase of 40% in UTS and YM only in the direction of stress resulting in tissue to become anisotropic.

All seven scaffolds were restrained by suturing them onto metal grids. Scaffolds were sutured to grids without cells as controls and the same parameters were calculated as in the previous chapters. Below, the effect of restraint on all seven scaffolds is compared. It was hypothesised that simple restraint will have no effect on the integration of cells with scaffolds, or on the mechanical properties of the cell populated scaffolds. However, it was hypothesise that, collagen and ECM production will be increased on all the scaffolds.

7.2 The effect of restraint on the production of tissue engineered prostheses results

7.2.1 Cell attachment

Fig 7.2.1.1 shows the metabolic activity of fibroblasts on the seven scaffolds under restraint. The most surprising finding is the increase in metabolic activity of cells on PPL making it the second best scaffold. Fig 7.2.1.2 shows the difference at 14 days between scaffolds with and without restraint. Restraint only led to a significant improvement of the metabolic activity of cells on PPL. The other scaffolds showed some change but these were not significant.

DAPI images were not different for scaffolds with and without cells except for PPL. These showed that fibroblasts were capable of attaching to mesh intersections when the PPL was restrained (Fig 7.2.1.3).

7.2.2 Tensiometry

The effect on the mechanical properties of the addition of cells on restrained scaffolds is shown in Figs 7.2.2.1, 7.2.2.2 and 7.2.2.3. PD showed a significant decrease in UTS and UT strain. The only other change with the addition of cells was the decrease in UT strain in Th PLA with the addition of cells.

The effect of restraint on the mechanical properties of scaffolds with cells is shown in Figs 7.2.2.4, 7.2.2.5 and 7.2.2.6. Restraint only affected the UTS and UT strain of CD. YM was unaffected by restraint on all of the scaffolds.

7.2.3 Contraction

While many of these scaffolds contracted both with and without cells, scaffolds restrained with sutures did not show any significant contraction over 14 days of culture. Visual inspection of the scaffolds revealed no pulling through of the sutures through the material. Fig 7.2.3.1 shows the negligible contraction of scaffolds in restraint compared to scaffolds without restraint. Table 7.2.3.1 shows the significant differences between restrained and unrestrained scaffolds.

Scaffold	% Contraction without restraint	% Contraction with restraint	P value of difference in contraction
AL without cells	2	0.15	0.4
AL With cells	3.4	2.4	0.67
CD without cells	7.7	0.91	0.04
CD with cells	17.6	2.43	<0.001
PPL without cells	1.53	0.33	0.31
PPL with cells	1.18	0.44	0.38
PD without cells	4.38	0.4	0.055
PD with cells	7.17	1.7	0.044
SF without cells	4.24	0.37	0.1
SF with cells	2.26	0.32	0.38
SIS without cells	14.02	-0.45	<0.001
SIS with cells	15.4	-0.87	<0.001
Th PLA without cells	10.9	1.56	0.003
TH PLA with cells	14.1	3.14	0.044

Table 7.2.3.1. Difference in contraction of scaffolds with and without restraint.

Thus for the four scaffolds that contracted while unrestrained (CD, PD, SIS and Th PLA), the suturing of these scaffolds for 14 days significantly reduced this contraction (Table 7.2.3.1).

7.2.4 Matrix production

7.2.4.1 Total collagen production

Total collagen produced by cells on the seven scaffolds was calculated by standardising each scaffold (stained with Sirius red) with its controls. Fig 7.2.4.1.1 shows there was no significant change in collagen production assessed by elution of Sirius red.

7.2.4.2 Immunostaining for collagen I, III, IV and elastin

As before, collagen I, III, IV and elastin were assessed by staining with a fluorescent labelled antibody. Three postgraduate researchers who were presented the images for blind scoring marked each image using the scale below (n=9). In Table 7.2.4.2.1 we present the modal value and mean of the three researcher's scores.

Figs 7.2.4.2.1, 7.2.4.2.2 and 7.2.4.2.3 show the difference between the ECM products collagen I, III and elastin respectively on restrained and unrestrained scaffolds. We note only small differences in collagen I production on PPL and SF, and collagen III on SF which were improved by using restraint. However, the extent of the increases in the ECM proteins were small and were not statistically significant.

	Collagen I		Collagen III		Collagen IV		Elastin	
Material	Mode	Mean	Mode	Mean	Mode	Mean	Mode	Mean
AL without cells	++	1.67	+	0.78	-	0.33	-	0.22
AL with cells	++	1.89	+	0.56	-	0.33	-	0.33
CD without cells	++	1.78	+	0.56	-	0	-	0.11
CD with cells	++	1.67	+	1	-	0.33	-	0.15
PPL without cells	-	0	-	0	-	0	-	0
PPL with cells	-	0.44	-	0	-	0	-	0
PD without cells	-	0.33	-	0	-	0	-	0
PD with cells	+	0.56	-	0	-	0	-	0
SF without cells	-	0.22	-	0	-	0	-	0.22
SF with cells	+	1	-	0.33	-	0	-	0.33
SIS without cells	-	0.67	+	0.56	-	0	+	0.67
SIS with cells	++	1.67	+	1.1	-	0	+	1.22
Th PLA without cells	-	0	-	0	-	0	+	0.67
Th PLA with cells	++	1.78	+	0.78	-	0	+	1.44

Table 7.2.4.2.1 Summary of immunostaining results for restrained scaffolds. Modal and mean values are presented as reported by three blinded researchers (n=9). Qualitative and quantitative grading scale; - absent= 0, + mild presence=1, ++ good presence =2, +++ abundance =3.

7.2.4.3 Use of SEM to assess the extent of ECM production

As for the assessment of ECM by immunostaining three postgraduate researchers assessed the presence of ECM on the seven scaffolds. These are presented as the modal and mean values in Table 7.2.4.3.1 and these are compared to unrestrained scaffolds in Fig 7.2.4.3.1. Restraint led to increased ECM production on PPL. Representative images of new ECM on PPL are shown in Fig 7.2.4.3.2.

Material	Mode	Mean
AL without cells	++	2
AL with cells	+++	2.67
CD without cells	++	2.22
CD with cells	+++	2.56
PPL without cells	-	0
PPL with cells	+	0.89
PD without cells	+++	2.78
PD with cells	+++	2.67
SF without cells	++	1.89
SF with cells	++	1.89
SIS without cells	+	1.11
SIS with cells	+	1.44
Th PLA without cells	-	0.22
Th PLA with cells	+	1.22

Table 7.2.4.3.1. Summary of SEM results for restrained scaffolds. Modal and mean values are presented as reported by three blinded researchers (n=9). Qualitative and quantitative grading scale; - ECM absent= 0, + mild presence=1, ++ good presence =2, +++ abundance =3.

7.3 Discussion

The use of restraint during the culture of fibroblasts on seven scaffolds has only shown limited benefit. Fig 7.2.1.2 shows only one significant change, on PPL, to the metabolic activity of cells from all the scaffolds. The reason why PPL performs significantly better with restraint may be due to the stretching and fixing of the intersections of the PPL. This is where the cells were seen to congregate when stained with DAPI (Fig 7.2.1.3) and additionally new ECM was seen here too with SEM (Fig 7.2.4.3.2). Langer *et al*. have shown that fibroblasts prefer to grow on mesh nodes, lighter mesh filaments (<50g/m^2) and also that large pores may be too large for fibroblasts to bridge, in addition a pore size of <130µm was thought to lead to difficulties for fibroblast attachment [513]. The PPL mesh we used has a reported thickness of 0.1mm and a weight of 44g/m^2 with a pore size of 5.82mm^2 [288]. This large pore size was too large for fibroblasts to cross but they did manage to cross the smaller pores between filaments at the mesh nodes. We hypothesise that the use of restraint may have affected the geometry of the pores between filaments and not the macroporous pores which allowed the fibroblasts to attach.

In addition, increases in metabolic activity may be due to localised niche pressures. It has been shown that fibroblasts exert a pressure on the filaments to which they are attached and this pressure will be dissipated if more fibroblasts are present [514]. Current thinking is that it is not only the pore size but also the micro-environmental stress which may affect cellular attachment and proliferation on meshes.

The UTS and UT strain of CD, was increased by the use of restraint and cells (Figs 7.2.2.4 and 7.2.2.5). This may be due to reduced degradation of the scaffold as there was not an increase in collagen or ECM production. In turn this may also be a result of the stress placed on the microenvironment of the cells within the scaffold. With the exception of PPL there were no changes in ECM production on the scaffolds and therefore the null hypothesis is rejected.

Although the literature has shown improvements in collagen and elastin production with fixed strain [512] [508], we did not see any with our scaffolds except for PPL. The reasons for this may be that both of these research groups used a self assembled matrix scaffold, synthesised by dermal fibroblasts, which may be more amenable for cells to work with and align to. Also the axis of strain was uniaxial in their experiments but we used a biaxial strain in all four corners. The biaxial strain in our scaffolds was less than 10% at the time of suture. We expect the stress on the scaffolds to increase with time during culture due to the restriction to contraction seen with the matrices under restraint.

Contraction was significantly inhibited on unrestrained scaffolds with greater than 5% contraction (Table 7.2.3.1) leading to all restrained scaffolds contracting less than 3%. Therefore, when *in vitro* scaffold contraction is a perceived problem, restraint would be advocated. Whether scaffolds continue to contract post release of restraint forms an important question for *in vivo* study. It is postulated that the answer may lie with whether the scaffold is implanted *in vivo* in a restrained or unrestrained manner. It may be that scaffolds that were allowed to contract *in vitro* and have reached a stable amount of contraction, as witnessed by the fact that most contraction was complete within the first seven days (Fig 5.3.1), do not contract further *in vivo*. Contrastingly, it may be that fibroblast behaviour on restrained scaffolds is altered to prevent them from attempting to contract a fixed matrix and thus implanted restrained matrices do not contract further. These are interesting questions for an *in vivo* study.

In conclusion, the use of restraint, imparting unmeasured static stress on scaffolds, does not lead to significant improvements in cell attachment, ECM production or in the mechanical properties for five of the seven materials tested. PPL did show some benefit in terms of cell attachment and ECM production and the UTS and UT strain of CD became closer to native tissue.

We will now investigate dynamic strain as this is an evolving art and has been shown in some studies to be beneficial. As discussed further in chapter 10, it is becoming clear that we have identified two matrices, SIS and Th PLA that have shown the greatest potential as scaffolds to take forward for further evaluation for a prosthesis for use in SUI/ POP. The work in this chapter and the next assess the physical culture conditions for a tissue engineered prosthesis and chapter 9 assesses chemical culture conditions. The next chapter will describe the development of a variable stress rig commencing with Th PLA and repeating the work with SIS if the concept proves feasible and provides promising results.

Chapter 8 The effect of variable stress on a thermoannealed PLA tissue engineered prosthesis

8.1 Introduction

The use of a variable stress rig to improve the mechanical properties of tissue engineered constructs has been reported in the literature [508]. Most commonly a bioreactor is utilised. These have the disadvantage of being expensive and difficult to set up. The type of bioreactor and type of stress applied to each scaffold largely depends on the application. It has been shown that in order to create a tissue similar to native tissue, the stress applied to the scaffold should resemble that of the native tissue [515]. For instance, in engineering of vascular tissues a biaxial device has been used [516], whereas heart valve engineering requires a pulse flow or cyclic stretch [511].

In light of this literature (and the knowledge that mechanical conditioning is often used in the development of hard tissues such as cartilage and bone) it seemed reasonable to examine the effect of mechanical stress on the development of a tissue engineered material for pelvic floor repair. It is hypothesised that such stresses might induce the production of ECM proteins more characteristic of the native tissues.

To create a tissue engineered prosthesis for use in SUI/ POP, it may prove necessary to apply multiaxial stress. The stress intensity and frequency may need to replicate that of a female pelvic floor (Fig 8.1.1). No reports, were found, in the literature which have modelled the stresses and strains placed on the individual components of the pelvic floor of women daily. In particular there are no reports of the contribution of stress on the pelvic tissue which is repaired during SUI/ POP surgery.

However, Ashton-Miller and DeLancey have shown that the average area of the female pelvic floor is 94cm^2. The pressure on the pelvic floor is postulated to be 37N whilst standing, 19N during lying down and 129N during coughing [274]. It is not known what stress the endopelvic fascia is under during rest and daily activities as the stress will be divided between the various pelvic floor support structures. Therefore a variable stress system should be designed to allow the application of varying stress loads to the tissue engineered prosthesis.

In this part of the work the aim was to create a simple variable stress rig to obtain the proof of concept of whether a tissue engineered prosthesis for use in SUI/ POP responds positively to variable stress during culture. For this purpose a very simple variable stress rig was developed

(Fig 8.1.2), and tested with Th PLA as this had shown to be a good scaffold for a tissue engineered prosthesis. Briefly, the rig consisted of the scaffold being fixed in an inverted Scaffdex™ ring with 0.261g X4 weights being applied and removed on an alternate day basis. It was hypothesised that mechanical conditioning of cells on scaffolds would affect the proteins produced by the cells as a response to the stress applied and this would consequently affect the mechanical properties of the scaffolds.

8.2 The effect of variable stress results

The first variable stress rig (variable stress 1) showed that cells were not proliferating on the inverted Scaffdex™ rings (Fig 8.2.1). This was even with the scaffolds that were simply restrained without the application of weights. The mechanical properties were found to be unchanged from controls and there was no collagen production (data not shown). This was believed to be due to the height of the Scaffdex™ rings when inverted, which reached the top of the six well plates. Therefore, the cells were essentially at an air liquid interface. In addition, it was found that air bubbles would form under the rings thereby preventing media from reaching the cells.

Building on these initial findings improvements to the variable stress rig (variable stress 2) were made, as shown in chapter 2 (Fig 2.11.2). They involve cutting the ring in half from its base and using the ring the correct way up. This lowers the scaffold in to the media and allows air to escape from beneath the scaffold. The remainder of the chapter discusses the findings from the variable stress 2 rig.

8.2.1 Cell attachment

The use of Th PLA in the variable stress 2 rig without the use of weights led to a decrease in the metabolic activity of cells compared to unrestrained Th PLA scaffolds (Fig 8.2.1.1). The addition of weights to the Th PLA scaffolds led to a non-significant decrease in metabolic activity. Although there were decreases in metabolic activity compared to unrestrained scaffolds, there was still an increase over the 14 days of culture relative to day 0.

Looking at the DAPI images (Fig 8.2.1.2) we see a greater number of cells on both the restrained and variable stress 2 rigs than on the unrestrained scaffolds at 14 days. We did not find any areas where cells were absent on the scaffolds in the variable stress 2 rig which shows that the weights were not compressing and killing the fibroblasts.

8.2.2 Contraction

No significant contraction of scaffolds in the restrained and in the variable stress 2 rig was found (data not shown). This is because in both cases the scaffolds are restrained and therefore unable to contract.

8.2.3 Tensiometry

The UTS of Th PLA is increased by the use of variable stress (Fig 8.2.3.1), however this was found not to be significant. The UT strain (Fig 8.2.3.2) and YM (Fig 8.2.3.3) of Th PLA in the variable stress 2 rig was found to be unchanged.

8.2.4 Matrix production

Sirius red staining showed increased collagen production by fibroblasts on Th PLA in the variable stress 2 rig (Fig 8.2.4.1). However, this was not significant compared to restrained ($p=0.08$) and to unrestrained Th PLA ($p=0.09$). Further studies with longer periods of loading are clearly indicated.

Immunostaining showed some increases in the appearance of collagen III (Fig 8.2.4.2) and Elastin (Fig 8.2.4.3). Collagen I remained unchanged.

8.3 Discussion

The results show that cells may be cultured on a variable stress rig made of a Scaffdex™ ring with weights intermittently applied to the scaffold. Although the metabolic activity of cells was reduced compared to cells on free scaffolds, DAPI images did not show a significant reduction in the number of cells. This implies that the cells do proliferate but are less active. In addition, no areas of the scaffold devoid of cells were found and so can conclude that the cells may be cultured beneath the weights without an adverse impact on cell proliferation.

Kim *et al*. have shown cyclical mechanical strain increased proliferation of smooth muscle cells and increased collagen and elastin gene expression [517]. The authors also showed alignment of cells to the direction of strain, an increase in UTS and YM. Interestingly, mechanical strain increased collagen and elastin production on the fibronectin-coated PGA scaffolds but not on the type I collagen sponges [517]. This is an important finding and tells us that cells behave differently on different scaffolds under strain and the findings from one scaffold cannot be extrapolated to another. In addition, it also shows that cells are not just responsive to macro-stress (stress upon the entire scaffold) but will respond to microstress, which is also dependent upon scaffold microarchitecture. Due to the confluence of cells on these scaffolds we were unable to see whether there was alignment of cells on the Th PLA. However, as we applied biaxial stress, alignment may not be noticeable as described by the authors above; who used uniaxial strain.

Gauvin *et al*. have shown an increase in UTS and YM of 40% by the use of dynamic uniaxial stress on a self assembled scaffold [508]. The UTS and YM perpendicular to the scaffold however decreased by 40-60% [508]. Therefore as biaxial strain was applied we may not have seen such differences in the mechanical properties of Th PLA by the use of our variable stress 2 rig as tissue would not become anisotropic. Grinell has shown that fibroblasts will orient their cytoskeleton in the direction of stress [462], and therefore in biaxial stress upon a randomly oriented scaffold cytoskeletal alignment may not occur. The mechanical properties of the scaffolds under variable strain remain close to those of native tissue.

Gilbert *et al*. have shown that the variation of the type of strain can affect the resultant tissue produced by fibroblasts on a SIS matrix [515]. Therefore, logically, it follows that to create a tissue for a specific function, replication of that function provides the fibroblasts with the correct signals to produce this tissue. The fibroblasts however, must be capable of producing this tissue. Oral fibroblasts increased collagen III and elastin production by the use of variable

biaxial strain. Unfortunately, this work was undertaken at the end of this thesis and time did not permit its completion. For example due to the breakdown of the electron microscope it was not possible to assess ECM orientation with the variable stress rig. This work will be completed after submission of this thesis and repair of the electron microscope.

Histological analysis of neo tissue architecture may reveal changes between scaffolds under varying conditions. In addition to this, the functional ability of fibroblasts has been shown to be altered by the use of cyclic strain and therefore the assessment of chemical mediators released by fibroblasts may give an indication of the various processes occurring during low and high strain. Also tissue engineered prostheses will be implanted under strain and will be required to take strain from day 0. Therefore if an *in vitro* rig can model this strain then early failure can be predicted from the *in vitro* results.

To conclude, a simple variable stress rig has been designed which allows cellular proliferation and ECM production. It has been shown that collagen production may be increased by this loading regime and that this affects the mechanical properties of the scaffold to a degree. This simple rig can be examined further and greater loading for longer periods of time is clearly indicated. These findings should enthuse researchers to a further avenue of research using mechanical conditioning of tissue engineered prostheses for POP/ SUI. We hypothesise that with the development of a rig capable of providing a higher frequency of stress change may lead to tissue more like native paravaginal tissue.

Chapter 9 The effect of Vitamin C on a PLA tissue engineered prosthesis

9.1 Introduction

Vitamin C (L-ascrobic acid/ ascorbate), has been shown to stimulate collagen production from skin fibroblasts via enhancement of type I and III collagen gene transcription [503]. The stable form of Vitamin C is Ascorbate-2-phosphate (Asc 2p). This has been shown to increase collagen synthesis and improve ECM organisation by skin fibroblasts in culture at a concentration of 0.1-1mM [518]. Ascorbic acid not only stimulates collagen production at the mRNA transcriptional level but also stimulates lysly hydroxylase which stabilises collagen by cross linking [519]. In culture, cells have been shown to devote 85% of synthesis to collagen I and 15% to collagen III, in addition to small amounts of type IV and V collagen. The optimum concentration of ascorbic acid to produce collagen by cells in two dimensional culture has been shown to be 30-40μM [519] [520].

Similarly, in three dimensional scaffolds Asc-2p has been used to stimulate collagen production. SIS sheets were seeded with dermal fibroblasts for four weeks [502]. The resultant composites were then cultured with media alone, media with 0.45 mM Asc-2p or fibronectin pre-treated SIS and 0.45 mM Asc-2p. Fibronectin treated scaffolds also treated with Asc-2p showed the most increase in type I collagen with more organised tissue on the surface of the SIS. There was no change to the matrix metalloproteinase activity which suggests the SIS scaffold was not being broken down over the four weeks [502]. Other researchers have also utilised the potential of vitamin C to increase collagen production. Gauvin *et al*. used 0.3mM ascorbate to increase collagen production by fibroblasts subjected to dynamic strain [508]. Proulx *et al*. also utilised 0.3mM of ascorbate to increase collagen production by corneal fibroblasts [521]. Guo *et al*. utilised 1mM ascorbate to increase collagen production from corneal keratinocytes [522].

A literature search was unable to find any studies investigating different concentrations of ascorbate in the production of tissue engineered prostheses. Therefore the effect of 0.3mM, 1mM and 3mM ascorbate compared to no ascorbate in the production of collagen from oral fibroblasts on a PLA scaffold was investigated. The effect this had on the other properties of the scaffolds including cell attachment, contraction and biomechanical properties was also assessed. It was hypothesised that ascorbate would lead to increased collagen production without affecting the other parameters of the tissue engineered prostheses.

9.2 Results of the effect of Asc-2p

9.2.1 Cell attachment

Fig 9.2.1.1 shows the effect of vitamin C on the metabolic activity of cells. Both 0.3 and 1mM of Asc-2p led to a significant increase in metabolic activity at 14 days of culture. DAPI images showed increased numbers of cells with 0.3 and 1mM Asc-2p (Fig 9.2.1.2).

9.2.2 Tensiometry

Asc-2p led to an increase in the UTS of PLA (Fig 9.2.2.1), however, only 0.3mM was significant. The addition of cells did not significantly increase the UTS. The UTS was at the lower limit of native tissue. The UT strain (Fig 9.2.2.2) and YM (Fig 9.2.2.3) were inferior to native tissue and did not change significantly with the addition of Asc-2p.

9.2.3 Contraction

Neither the addition of cells nor ascorbate led to significant changes in the contraction of PLA, which was approximately 30% over 14 days (Fig 9.2.3.1). Looking at Fig 9.2.3.1 one can see that the majority of contraction occurred during the first seven days of culture.

9.2.4 Total collagen production

Cells cultured with 0.3mM Asc-2p produced significantly more collagen than cells with no Asc-2p as assessed by absorbance of Sirius red stain (Fig 9.2.4.1). The production of collagen in the presence of Asc-2p showed a peak at 0.3mM which decreased at higher concentrations. The extent of the increase with 0.3mM compared to no Asc-2p was 60% over the 14 days of culture.

9.3 Discussion

Asc-2p led to significant increases in cell metabolic activity, UTS and collagen production on a PLA scaffold. There were no significant changes to the UT strain, YM and contraction of the scaffolds. The mechanical properties of PLA are inferior to those of native tissue and therefore prostheses based on PLA without thermoannealing would not be recommended for use in SUI/ POP.

However, as a proof of concept study, these experiments show the benefit of chemical modulation; in this case ascorbate. Asc-2p not only led to increased collagen production as hypothesised but also increased cell metabolic activity. This may have been due to the increase in collagen production, however, looking at the images in Fig 9.2.1.2 it is seen to increased cell proliferation too. This may have been due to a direct effect of the Asc-2p on proliferation or the production of increased collagen may have led to an increased surface area for fibroblasts to proliferate and attach to.

There was an optimal increase in total collagen production with 0.3mM Asc-2p. It would have been interesting to see what type of collagen ascorbate stimulated and whether this was at the expense of other proteins such as elastin, however, these experiments were conducted prior to obtaining the immunofluorescent antibodies and thus it was not possible to do this. Other researchers have shown that ascorbate may lead to a reduction in elastin production from skin fibroblasts [523] or elastin and lysyl oxidase from smooth muscle cells [524]. This may explain why the UTS is affected and the YM remains unchanged. The important message still lends to the concept of chemical modulation to increase cell proliferation and collagen production which may in turn improve some of the mechanical properties. However, one must be aware that improvement in collagen content may be accompanied by a reduction in the production of other cellular proteins. In addition, other properties of the graft may be affected, for instance an acidic environment may lead to quicker hydrolysis of PLA.

Other researchers have used ascorbate to increase collagen production [502] [508] [521] [522] but there are no reports of increased cell proliferation or improvements in UTS with its use. Soucy *et al*. have shown a doubling in collagen I production by lung fibroblasts with the addition of 500µg/ml ascorbate which did not affect the YM [525]. Grinnel *et al*. have shown increased crosslinking of collagen fibres with the use of ascorbate but did not assess the mechanics of the new tissue [526]. Increased cross linking may be the reason why an increase in the UTS with the addition of Asc-2p was seen. Throm *et al*. used epidermal growth factor to increase collagen I content produced by foreskin fibroblasts over 21 days culture but again this did not affect the UTS [526].

Other cells have been manipulated by ascorbate and/ or other chemical mediators. The use of ascorbate with transforming growth factor-β was shown to increase cell proliferation of Mesenchymal stem cells on a scaffold [527]. Nugent *et al*. have shown that chondrocytes may be stimulated to increase GAGs and hydroxyproline production [528]. Our results and those reported in the literature point to the important findings that chemical mediators may be used to alter cell proliferation, collagen production and mechanical properties. The optimum constituents of culture media will depend largely on the native tissue requirements both histologically and structurally and it would be prudent that investigations assessing the utility of these chemicals assess these in future experiments.

Chapter 10 Discussion

10.1 Failure of current prostheses

The failure and complications associated with current prostheses for pelvic floor disorders and their impact on patient health and quality of life was the motivation for pursuing a higher research degree in this field. After a comprehensive review of the literature it became apparent that our knowledge of why prostheses fail is still in its infancy. A particular problem is the lack of long term follow up studies. The newer less invasive procedures using lighter meshes for SUI, and even the inclusion of mesh for POP surgery, all obviously lack long follow up data in sufficiently large populations resigns us to this lack of knowledge. Nevertheless, patterns for failure have emerged and we have categorised these in Fig 10.1.1 for synthetic meshes, Fig 10.1.2 for autologous grafts and 10.1.3 for biological grafts.

For all meshes, early surgical complications are shown in the grey boxes. The causes of the common post-operative problems; bladder outflow obstruction, erosion, pain and failure are shown in the green boxes. The figures relate to SUI procedures as this is better studied than POP and also the SUI technique is standardised and therefore more comparable. We have discussed the relevant data of the outcomes in POP surgery in the text.

The reported success of mid urethral tapes depends largely on the definition of success; these encompass both objective and/ or subjective evaluation. The longest prospective follow up data is of 11 years from Nilsson *et al*. who reported 90% objective success rates and 77% subjective success rates with TVT [529]. Other studies reporting medium term outcomes have shown lesser success rates of 81% and 70% at five years [530] [531]. Nilsson *et al*. also reported no cases of erosion over 10 years, whereas other studies have shown a rise in incidence of erosion over time [532], which also varies with the type of tape utilised [533]. Mesh erosion may occur within weeks to years after surgery [534]. The rate of mesh erosion for POP is even higher than SUI surgery at 8-24% for anterior repair, 6-13% for posterior repair and 3.4-16% for apical repair [535]. The success rate of POP procedures has been shown to be improved by the use of synthetic mesh by 15% by Jia *et al*. [213] and as reported more recently in a Cochrane systematic review [536]. However, the risk of complications increases as discussed above.

The aetiology of bladder outflow obstruction post tape surgery is hypothesised to be linked to haematoma formation, infection and the host response to the material [537]. PPL has been shown, in some cases, to undergo degradation by the host immune system particularly in the presence of infection. This leads to pro-inflammatory breakdown products of the PPL resulting in further inflammation and mesh prominence leading to pain. If the vaginal epithelium is breached it is termed extrusion. Violation of the urinary tract mucosa is termed erosion and is a much more serious complication. If the fibrotic response to the material leads to tissue thickening this is thought to lead to varying degrees of bladder outflow obstruction.

Infection and the host inflammatory response may explain why some patients develop erosion. In addition, if the mesh has a higher YM than native tissue and fails to integrate and stimulate new tissue formation then there will be a continuing grating effect leading to the mesh wearing through the host tissue which may be responsible for pain and dyspareunia. It has been suggested that fixing the mesh will make it less likely to contract and “grate” through host tissue. The biomechanical properties of meshes in relation to success and erosion rate have been reviewed by us in section 1.10.5 [538]. We found no simple correlation between the mechanical properties of prostheses and their success and erosion rate but emphasise there is a paucity of data in this regard.

As discussed in chapter five the significance of synthetic mesh folding or contraction is still poorly understood. There is no evidence relating mesh contraction to any of the reported complications. However, as this is a feature of meshes it should still be recognised and will require further study.

The host response to PPL was previously considered to be fibrotic leading to no degradation of implants. However, more recently, it has been proposed that this is not necessarily the case and the immune response may be individual to each person and this may also be altered by infection [537].

The current response to PPL is understood to occur within minutes of host injury whereby a biofilm is formed on the “inert” synthetic implant, proteins are adsorbed onto this film independent of a cell response known as the Vroman effect [539]. Proteins such as albumin, fibrinogen, immunoglobulins, kinninogen and ECM molecules are adsorbed on to the biofilm. An inflammatory response, including leucocytes, macrophages, the complement system, the clotting and fibrinolytic cascades ensues against these bound proteins, in particular fibrinogen [540] [541]. Bacterial infection at this time may affect the biofilm leading to an altered immune response [542].

Thereafter, a more typical chronic inflammatory response will follow i.e. a foreign body reaction, with formation of granulation tissue with fibroblasts, macrophages, granulocytes and giant body cells with some neovascularisation. Neutrophils generally only persist for the first stage of the response. They produce a cytokine profile dominated by tumour necrosis factor-α, interferon-ϒ and interluekin-1 referred to as the M2 response which stimulates T helper-2 (Th 2) cells and is pro-inflammatory [542]. Macrophages are postulated to mediate the inflammatory response based on their "acceptance" of the biofilm. This is when the material composition, pore size, weight, and structure of the implant become important [543].

The pore size, surface properties and weight are important factors for macrophage infiltration and later fibroblast infiltration and blood vessel formation [212]. Pores that are too large have been shown to be filled with fat and pores that are too small become encapsulated [544]. The ability of fibroblasts to integrate in turn allows new ECM to be produced by the fibroblasts. The implant surface architecture has been shown to affect the amount of tissue ingrowth [545]. The acceptance of the above factors by macrophages leads to varying degrees of chronic inflammation, tissue ingrowth and mesh integration as shown in Fig 10.1.4. Once fibroblasts are present, growth factors and cytokines stimulate the healing processes. The important cytokines are platelet derived growth factor, fibroblast growth factor, keratinocyte growth factor, epidermal growth factor, transforming growth factor- α, and transforming growth factor-β [546]. This will be altered in the presence of chronic inflammation.

Interestingly, the presence of a large number of macrophages has been shown to be associated with erosion [547]. In the five cases reported by Kavvadias *et al*. it was not clear whether the macrophages were contributory to the erosions or were a result of mesh infection. Bacterial contamination has been linked to mesh complications too [548]. However, mesh erosion in these patients was only linked to small bacterial counts ($1x10^3$) after enriched culture. Therefore the importance of a low grade bacterial infection modifying the implant biofilm and immune response is still not known.

Besides mesh related factors, surgical and patient factors are also significant and may be irreversible. Factors affecting wound healing such as age, menopausal status, smoking, concomitant illness, obesity, medications and the ability of host fibroblasts to produce an ECM may also affect the inflammatory and healing response.

It can be concluded that synthetic materials stimulate a chronic inflammatory response to varying degrees as depicted in Fig 10.1.4. The amount of host tissue infiltration is dependent upon mesh composition, pore size and configuration and this will in turn influence mesh integration and new ECM production. The importance of bacterial infection in the aetiology of

erosion is yet to be elucidated and the impact of the biomechanical properties on outcomes still requires further study.

The difference between the autologous sling procedure and the other procedures is the donor site morbidity which involves infection, pain and scarring which are most often self limiting. In addition the former procedure takes longer and also leads to increased hospital stay [549]. The rate of post operative urinary retention is also higher with autologous sling procedures due to the tension applied during the surgical procedure [139]. Albo *et a*l. have shown 6% of women required surgery for bladder outflow obstruction within two years of sling surgery [139]. The catheterisation rate one year post surgery has been shown to be 10% [223]. The most likely reason for voiding difficulties in patients post autologous sling surgery is likely to be the technique which involves tension via suture/ "string" fixation. The correct tensioning of slings is currently not an exact science and relies heavily on surgical experience. The formation of a haematoma at the surgical site may also contribute to post operative "tightening" of the autologous sling. However, one would not anticipate a haematoma in such a high proportion of patients inferring that this is not the only contributory factor. No hyperinflammatory response has been reported with autologous tissue and therefore it is unlikely that this contributes to the high obstruction rate either.

The rate of erosion with autologous slings is extremely low and is only described in a few case reports being diagnosed from a few weeks to five years after surgery [550] [551] [552] [553]. However, reading the case reports it is apparent that all patients had symptoms of pain or bladder irritation early (with a few weeks) after their surgery. Therefore it is feasible that erosion is an early feature in patients after sling surgery and the diagnosis may be made at any point thereafter. This suggests that it may be early infection or a lack of tissue infiltration early in the postoperative period that leads to failure of integration and erosion. However, there is no histological data to support this hypothesis. A biomechanical mismatch as an aetiological factor is also considered less likely as the risk of erosion would then be expected to continue over a long time course.

The early success rate of autologous sling procedures have been shown to be very good [223] but long term data is lacking and shows a large variation ranging from 90% to 53% success rates depending on the definition of success [554] [223]. Woodruff *et al*. have shown in five women who failed SUI surgery that there was moderate degradation of the autologous fascia which remained intact and the tissue did show moderate fibroblast infiltration and vascularity [214]. Therefore it may be the progression of tissue weakness, graft degradation, failure of the mesh to stimulate new ECM production that leads to failure with autologous grafts.

Autografts stimulate a very limited foreign body reaction and generally are thought to integrate well into native tissue [555]. The autograft contains a native tissue matrix with its intervening cells (mostly fibroblasts). The quality of both the scaffold and the cells is largely patient dependant. Therefore elderly postmenopausal women with medical comorbidities are likely to provide weakened autografts. Also after the absorption of the scaffold the replacement tissue is likely to be weak in these patients. Although Woodruff *et al*. have shown graft degradation in failures, Fitzgerald *et al*. have not shown large degradation of rectus fascia slings in successful procedures and have shown remodelling with fibroblast proliferation, neovascularisation and ECM production [222]. This suggests that tissue degradation may be the cause of failure in susceptible patients.

The other reason for failure is progression of host tissue laxity and failure to produce new ECM. Almeida *et al*. have shown that PPL and SIS stimulate greater inflammatory responses than autologous tissue and by day 30 the inflammation in the latter was equivalent to a sham surgery group without any implant [556]. Autologous tissue was also shown to lead to less collagen III production compared to SIS and PPL and this may be the reason for its failure. It may in fact be the case that it is the lack of an immune response initiating constructive remodelling of new collagen production that is responsible for failure. Fig 10.1.5 shows a schematic of the current understanding of autologous grafts.

Biological grafts may be of cadaveric or xenogeic origin. The failure rates are individual to the type of graft material used. Cadaveric fascia has poor medium term success rates (61-48%) after 12 months, which is the case for both SUI [227] [229], and POP surgery [228] [230]. Frederick *et al*. have shown 56% of failures occur after 12 months [315]. Woodruff *et al*. have shown moderate levels of graft degradation and only peripheral infiltration of cadaveric grafts by fibroblasts in patients where grafts failed [214]. Van de Vord *et al*. have shown in rat models, that cadaveric dermis forms a thick capsule around the graft which persists to 12 weeks and limits fibroblast infiltration and therefore implies a persisting inflammatory phase [320]. This was also supported by the finding of increased inflammatory cells. Erosion rates vary between 0% [180] [557] reported up to four years to 11% [232]. In addition to this cadaveric tissues have the added risk of virus or prion transmission albeit this is small (1 in 8 million).

Porcine dermis however, showed no collagen capsule formation and showed very little cell infiltration and angiogenesis [320]. Woodruff *et al*. reported total encapsulation of the porcine dermis graft with no cellular infiltration in a patient who failed [214]. Clinically, PD is shown to have failed in 39% of women within one year of surgery [558]. The reasons for poor

incorporation of PD may lie with the processing method, which may alter the native biological matrix sufficiently to affect its incorporation [432]. It appears that PD stimulates very little inflammation, does not attract fibroblasts and is eventually encapsulated and/ or degraded by a foreign body response [559].

In contrast, SIS stimulates a vigorous inflammatory response, and has shown a thick capsule at two weeks, thereafter this was remodelled and fibroblasts and new blood vessels were seen to be integrating into the mesh [320]. At 12 weeks the implant was indistinguishable from the native tissue. Again the processing methods and the extent of decellularisation will affect this outcome. SIS has been described to be remodelled by a "constructive" process [560].

Remodelling begins with an intense inflammatory response which leads to occasional seroma formation. Badylak argues that this is part of the early remodelling process and is driven by blood monocytes [561]. Occasionally infection intervenes leading to purulent discharge [562]. SIS has been shown to contain fibroblast growth factor-β and transforming growth factor-β which are postulated to reduce the inflammatory reaction [563]. Polymorphonuclear cells are most prominent up to the 14 days at which point macrophages peak [564]. Thereafter, macrophages remain at a lower stable level and are thought to promote constructive remodelling. Foreign body giant cells are present for up to 30 days but have been shown to be absent at 90 days.

Macrophages have been shown to carry the cell surface marker CD163+ which classifies them as having a remodelling phenotype (M2). However, the M2 response will interchange with the proinflammatory (M1) response during different time points depending on the processing of the SIS [435]. Therefore Badylak argues, that the recognition of a proinflammatory phenotype (M1) over a tissue remodelling phenotype (M2) requires the mapping of cell surface markers and cannot be done by simple histological assessments which are most commonly reported. It must be understood that the M1 and M2 responses lie on a continuum and are not distinct entities[334].

SIS topography and microarchitecture has been shown to favour cell attachment [565]. Also SIS degradation has been shown to release vascular endothelial growth factor and fibroblast growth factor-β which promote vascularisation. In addition SIS degradation products have been shown to be chemotactic stimulating new cell influx as scaffold degradation occurs [566]. The presence of cellular material or cross linking in addition to xenogeneic ECM epitopes are thought to affect the immune response. One such epitope identified as causing hyperacute rejection is the α-Gal epitope [567], although in primate models there has been no evidence of rejection [568].

Regardless of the macrophage phenotype the ultimate outcome of tissue integration and the strength imparted to the native tissue is clearly what is most important. Claerhout *et al*. have shown that SIS was remodelled into a thin fibrotic layer within three months in a rat abdominal wall model [559]. Ozog *et al*. showed that SIS was remodelled into collagenous tissue with good vascularisation but this added no strength to the repair and at 90 days was weaker than a native tissue repair [564]. Dora *et al*. have shown graft weakening of cadaveric dermis, porcine dermis and SIS too in rabbit models up to 12 weeks [300].

Clinical results from the use of SIS are limited. Short term results with SIS augmentation in anterior repair have shown 86% anatomic cure rates but these did not translate to superior quality of life results[235]. Chaliha *et al*. used SIS graft to augment anterior repair and found better results than native repair at six months but at two years this difference was no longer apparent [569]. Long term results were recently reported with SIS used as a pubovaginal sling in 48 patients [570]. The authors reported 69% cure rates at a median of 76 months follow up, with no dyspareunia, retention or erosion.

It can be concluded that although SIS has the ability to integrate well, support and attract host fibroblasts and promote vascularisation of tissue, it is mostly degraded by three months and may not leave behind a tissue with sufficient strength for the repair to remain successful. This is specifically a concern when considering success of SUI/ POP procedures. In addition, the native cells that may be attracted may indeed be the ones causing the tissue laxity in the first place and therefore recruitment of these will not logically be beneficial. New ectopic cells which have the ability to produce good ECM may provide the answer. Fig 1.10.6 shows the schematic of the host response to biological grafts.

10.2 Solutions for an improved prosthesis

Current thinking of the failure of prostheses for POP/ SUI surgery is summarised in Figs 10.1.4, 10.1.5 and 10.1.6. It can be seen that the problems lie with:

1) A poor inflammatory response- which leads to encapsulation and failure
2) Poor tissue integration- which leads to failure and/ or erosion
3) Persistence of chronic inflammation (M1 response)- which leads to erosion
4) A lack of continued ECM production after the constructive remodelling (M2 response)- which leads to long term failure

Therefore, a successful implant will need to stimulate a sufficient inflammatory response to prevent itself from being encapsulated and thereafter allow tissue integration. It is our opinion that the persistence of a small proportion of chronic inflammation with a synthetic scaffold is not necessarily harmful (purple arrow Fig 10.1.4) and allows the pelvic connective tissue to retain its strength. With a permanent scaffold this chronic inflammation will persist lifelong and if native tissue weakens, or an infection increases the inflammation, then erosion may occur. There is just not enough data to say how much chronic inflammation is acceptable and whether the risk of erosion increases much more over time. With a biological scaffold it is currently suggested that the M2 response is preferred but with complete tissue integration and graft degradation there is no longer a stimulus to continue new ECM production. In addition the native cells have been shown to produce substandard ECM and cannot be relied upon to remodel pelvic connective tissue with good ECM.

Our solution was to develop a tissue engineered prosthesis using a scaffold with autologous cells. It has been shown that the use of autologous cells with scaffolds can lead to improved outcomes not possible with scaffolds alone or cells alone [571] [572]. Badylak has however, shown a shift towards the M1 proinflammatory phenotype in a rat abdominal wall model using cellular than cell free porcine urinary bladder matrix [573]. The significance of this is not known as the study was only conducted up to 28 days.

Contemporary work in the field of tissue engineering has mainly concentrated on strengthening the rhabdosphincter in women with stress incontinence. Laboratory models involving rats have shown benefits with the injection of autologous fibroblasts and muscle derived cells directly in to the urethral sphincter [574]. However, only the muscle derived cells led to increased contractility of the urethral muscle.

In the clinic, work from the Innsbruck laboratory reported good results with the injection of myoblasts and fibroblasts para-urethrally in women with SUI [241]. However, it has subsequently emerged that there were many scientific irregularities in their work and the majority of their publications regarding this have now been retracted [243] [575]. Another group in North America have reported a pilot study showing cure from SUI in one of eight women, with five being improved with the injection of muscle derived stem cells into the urethral sphincter [576]. Improvements were seen after a delay of three to eight months, suggesting the improvements may be due to a delayed bulking effect or restoration of the urethral sphincter [577]. However, no evidence of increased sphincteric activity has been yet presented.

Despite the work continuing on improving the urethral sphincter, it has long been accepted that in the majority of women with stress incontinence, connective tissue laxity and urethral hypermobility play a greater role than intrinsic sphincteric deficiency [578] [579]. Although, more recently DeLancey has questioned this and presented evidence that urethral hypermobility is difficult to assess and therefore has not correlated as strongly with SUI as has the mean urethral closure pressure [580]. However, DeLancey does accept that the majority of women will show improvement with a procedure to stabilise and support the urethra as opposed to methods which aim to increase sphincteric contractility. Therefore, a procedure to treat the connective tissue laxity component of SUI is entirely logical and also lends itself to POP surgery.

Development of tissue engineered slings is still at an early stage and has not yet reached the clinic. *In vitro*, Skala *et al.* [236], Langer *et al*. [237] and Contineza *et al*. [239] describe fibroblast isolation and culture with existing meshes and assess fibroblast attachment, Kapishke *et al*. [238], in addition, show ECM production on to the meshes.

In a rat incontinence model (sciatic nerve transection), Cannon *et al*. have shown improvements in leak point pressure (LPP) after the implantation of acellular and cellular SIS with muscle derived cells [240]. In a similar model, Zou *et al*. have shown increases in LPP with the use of a silk sling with Mesenchymal stem cells which led to increased collagen production at 12 weeks [581]. The UTS and YM of the tissue were also increased. Labelled Mesenchymal stem cells were seen at the implant site four weeks after implantation and improved the integration of the silk matrix.

The use of tissue engineering for POP has only been investigated to our knowledge by two studies. Hung *et al*. used vaginal fibroblasts with a high collagen producing potential on a PLGA scaffold [582]. A tissue engineered fascia was created by culture over five days in 10% fetal

bovine serum and 50ng/ml ascorbate. After transplantation transplanted cells were shown to be producing a neo fascia for up to 12 weeks. Although the authors showed that some women with POP have vaginal fibroblasts with high collagen producing potential, this therapy would be limited only to those women with vaginal fibroblasts capable of generating new collagen. Ho *et al*. have shown that muscle derived stem cells seeded on a SIS scaffold may differentiate in to smooth muscle cells up to eight weeks of implantation [583].

From the above we see that the progress made with tissue engineered prostheses for SUI/ POP is still at an early stage. The majority of studies have described isolation of cells and shown that they can be cultured on a scaffold. Three studies have shown the cells to produce ECM, two in a rat model. None of the reported literature, however has compared a range of scaffolds for these parameters. Neither have they discussed the ideal mechanical properties required from prosthesis for human use. The contraction of prostheses has not been reported, which is increasingly being recognised as important. Clearly, methods to improve the properties of scaffolds, either mechanical or chemical are important but they are currently lacking in the literature.

Therefore, a comprehensive approach, to investigating a tissue engineered prosthesis which may be used for treating SUI/ POP, was taken. Fig 1.6.7.1 in the introduction chapter shows the key inputs and outputs of such a prosthesis. In this thesis we evaluated the best scaffold from a selection of seven scaffolds and thereafter assessed different culturing conditions. All the work undertaken is *in vitro* and will inform our *in vivo* studies. A schematic of the target in vivo response is shown in Fig 10.2.1.

10.3 Relating results from this study to the current literature

For the first time in the literature a correlation between the mechanical properties of SUI/ POP prostheses with their success and erosion rates has been reported [538]. It was concluded that implant integration and the host response need to be considered, as well as the mechanical properties, to design materials with lasting clinical benefit.

The *in vitro* parameters which are important when designing a tissue engineered prosthesis were then sought. Thereafter seven scaffolds were assessed against these parameters and were graded (Table 10.3.1) to select the most appropriate to take forward to *in vivo* study.

Table 10.3.1 summarises the properties of the scaffolds. The scaffolds with the best properties are SIS and Th PLA. Restraint led to an improvement in the UTS of SIS and a deterioration of the UT strain and YM of Th PLA. The variable stress 2 rig led to a decrease in the metabolic activity of cells and showed similar mechanical properties to unrestrained Th PLA. Collagen III and elastin appeared to be increased and this suggests much more work is needed in light of these promising results. Also 0.3mM Asc-2p led to increased collagen production on a PLA scaffold. Although the current (non thermoannealed) PLA scaffold is poor biomechanically the ability of cells to improve this plus the addition of ascorbic acid needs further investigation. It is clear in this study that we have only touched the surface of how collagen stimulating agents such as ascorbic acid can improve mechanical properties of tissue engineered constructs.

From the parameters assessed, one would assert that the ability of a scaffold to deliver cells, capable of making new matrix, to the area of weak tissue is probably the most important. As discussed in section 10.1 it is a failing of current prostheses to stimulate cells to produce good quality collagen which leads to surgical failure and/ or erosion. Therefore it is postulated that it is only with the delivery of a sufficient number of cells capable of integration, proliferation and repair that long lasting success may occur.

Currently, it is not known how many cells are required in a tissue engineered prosthesis, whether these cells will require a well vascularised graft bed to take and whether these cells will persist long term to continue ECM production.

The first question can only be answered by a comparative *in vivo* study but one would propose that more cells would increase the chances of cell survival, proliferation and ECM production and therefore be more beneficial. However, cells that reach confluency may experience cell-cell inhibition and may differentiate into fibrocytes.

The second question is interesting and can be related to current autologous sling procedures, whereby a fascial sling is removed and ectopically transplanted with its residing cells. There have been no reports of problems with graft uptake or tissue necrosis implying that the sling becomes vascularised easily in the paravaginal bed. Therefore one would not expect a problem to occur specifically with a tissue engineered graft. Also in a rat SUI model, Cannon *et al*. showed no adverse inflammatory reactions to implantation of a cell seeded SIS matrix [240]. Muscle derived cells were implanted paravaginally and were shown to produce myotube structures by four and eight weeks culture. Other groups have shown the same findings and therefore this reassures us that the graft and cells will take [581] [582].

The final question has been investigated by Chang *et al*. who have shown diversity in fibroblasts from different sites [259]. In addition to this fibroblasts were noted to retain positional memory and if transplanted ectopically were shown to retain memory of their previous functional role over a number of generations [259]. This will mean a great advantage in terms of ECM production in a region which often contains fibroblasts with a reduced ability to produce good quality ECM.

More recently, it is being recognised that it is possible to change transdifferentiation pathways of fibroblasts by altering the culture conditions [584]. The authors suggest that by changing fibroblast commitment, it is possible to exponentially stimulate a tissue remodelling phenotype. This leads to the use of modified fibroblasts, which may be tailored to specifically produce ECM for the pelvic floor. The studies relating to this work are however, still at a preliminary stage and much more work is needed in this area.

At present it is not known how much contraction is required or acceptable from a pelvic organ prosthesis. Both Th PLA and SIS contracted to approximately 15%. This is similar to that reported for PPL [370] [304]. Contraction can be prevented *in vitro* by the use of restraint but the effect this may have on subsequent implantation is not known.

In the review of the mechanical properties of contemporary prostheses, it was shown that implantation led to a reduction in UTS in all the materials reported in the literature (Fig 1.10.4.1). The YM was more variable with implantation (Fig 1.10.4.2). Although, a direct correlation between UTS/ YM of prostheses with success/ erosion rates was not shown, these parameters are still important because, a prosthesis must have sufficient strength to provide support to the pelvic floor on implantation. All the materials tested in this study had the same or higher UTS than native tissue and would thus not have this problem.

In addition, the YM is related to the ability of the scaffold to stretch and as the scaffold is implanted it will be required to stretch immediately. If it resists stretch then erosion can occur through grating of native tissue. Another aspect of this, worth consideration, is the effect of stress shielding. This is well recognised in the orthopaedic literature [585]. The theory is based on having a stronger material next to host tissue. The stronger material takes the strain in the region and this consequently results in weaker host tissue which causes failure. Therefore integration of a prosthesis with similar mechanical properties to native tissue may also be important to prevent stress shielding.

More importantly, the two best scaffolds SIS and Th PLA are absorbable and the rate of degradation in relation to new tissue formation may dictate the failure rate. As the scaffold is degraded, new tissue needs to be formed to take the strain of the degrading scaffold. The scaffolds can be altered by cross linking and other methods to reduce their degradation rate but this may affect the host inflammatory response. Currently, it is not known what the ideal degradation rate should be and this will need assessment in an *in vivo* study.

The addition of cells to the scaffolds did not significantly affect the UTS (Fig 4.3.1) or YM (Fig 4.3.2). This may be because of the timescale of culture. Fourteen days may not have been long enough for *in vitro* scaffold degradation and although new ECM production in this timeframe has been shown, the neo ECM is probably too immature to have an impact on a scaffold which has a high baseline UTS. At present, the optimum culture time prior to implantation is unknown.

A culture period of two weeks was used and this has shown cell number increased on the Th PLA and SIS scaffolds over this time period. This was based on results from other work in the laboratory involving the urethra and skin [256] [586]. The key difference between the other applications and this one is that a stratified tissue is not required here. In addition, with the other applications once stratification and confluence was achieved constructs were implanted within a few days.

Whether immediate implantation after cell attachment is better will need to be assessed by an *in vivo* study. There are benefits to both a shorter and a longer culture period. The former reduces the risk of *in vitro* infection and will result in a reduced wait for a patient to undergo their procedure. In contrast, a longer time period allows cells to proliferate and commence ECM production. This will have implications for the host immune response. A longer culture period may also result in an alteration of the mechanical properties of the tissue engineered grafts, which was not seen over two weeks culture. This can only be answered by *in vivo* investigations.

Based on these results and others in the laboratory (unpublished work), one may envisage a window for implantation lasting from a few hours up to approximately four weeks after cell seeding. This provides the flexibility required by hospitals and surgeons. Therefore a patient may donate a small oral mucosa biopsy, from which fibroblasts are isolated and cultured (average two weeks). Thereafter these fibroblasts are seeded on to a matrix which is cultured between a few hours or up to four weeks. A patient's surgery can be arranged in that time period as shown by the red arrows in Fig 10.3.1. Further fibroblasts may be frozen and kept in storage and therefore if a repeat procedure is required may be utilised without the requirement for a further biopsy.

Preliminary experiments towards a proposed one stage approach to creating a tissue engineered prosthesis have also been commenced (blue arrows Fig 10.3.1). The data is not shown in the thesis as it has only reached a preliminary stage and thus far there is not enough data to draw meaningful conclusions. Early indications, however, are that there is outgrowth of cells from minced tissue but this is limited and will require investigation of techniques to encourage cellular outgrowth. This technique could remove the delay associated with cell culture and the expense of a clean room laboratory.

The production of new ECM consisting of collagen I, III and elastin was highest on SIS and Th PLA, although CD and AL contain these already and this makes it difficult to assess this on these two scaffolds. A key question that arises is whether this ECM is good enough and will it serve its purpose in strengthening a pelvic floor repair. The data regarding this in the literature is limited and we found no long term studies assessing the quality of tissue produced after transplantation of fibroblasts with a scaffold in to the pelvic floor. In the short term, Zou *et al*. have shown good collagen production by Mesenchymal stem cells on a silk scaffold which led to increases in UTS and YM of the tissues at four and 12 weeks [581], suggesting that good ECM was produced.

The ECM produced by oral fibroblasts will be more akin to autologous oral mucosa. Oral mucosa has not been shown to have weakened properties in women with SUI/ POP and therefore one would hypothesise that a good ECM will be produced. This is in contrast to bone marrow stem cells which may differentiate into the type of cells required by the particular region. The stimulus for stem cell differentiation may be physical or chemical [587] [588]. *In vivo* this will be dependent on the requirements of the particular region and therefore should, in theory, also lead to good ECM production.

In conclusion, for the first time superiority of two scaffolds (SIS and Th PLA) over five other scaffolds to attach fibroblasts, allow their proliferation and encourage new ECM production

has been shown (Table 10.3.1). Both SIS and Th PLA are biodegradable and therefore will not pose a long term risk of erosion. Their mechanical properties are also close to native tissue but as has been discussed in section 1.10.4 these may be affected by an *in vivo* response. Therefore, the key processes post implantation which will define the success of these tissue engineered prostheses are the inflammatory response, transplanted cell survival and continued ECM production to maintain good biomechanical properties of these tissues in supporting the weakened pelvic floor.

The use of simple restraint did not show a sufficient benefit to endorse its use with the scaffolds, except with PPL (which gave a poor performance overall). The use of a simple variable stress rig showed a non-significant benefit for collagen production and this should be investigated further. The use of Asc-2p also, in combination with putting tissues under dynamic tension while producing them in culture now needs to be examined.

This work establishes a foundation for future experimental paradigms and answers the question of which scaffold could be used and whether ECM production can be stimulated (by fixed or dynamic strain or the addition of agents which stimulate collagen production). The next section discusses on-going and future work which we hope will lead to a tissue engineered prosthesis which can improve the quality of life of the millions of women worldwide who require surgery for pelvic floor disorders.

10.4 On-going and future work

In the laboratory, work has commenced towards defining the ideal cell for the tissue engineered prosthesis. Early results have shown adipose derived stem cells work synergistically with fibroblasts to increase collagen and elastin production. This is also the case in the literature, where co-culture of stem cells with differentiated cells has shown that adipose derived stem cells may be stimulated to differentiate and work synergistically with the differentiated cells [589]. In addition, fibroblasts have also been shown to proliferate quicker in the presence of adipose derived stem cells [590]. The latter cells have also been shown to stimulate angiogenesis via distinct cytokine and protease expression [591]. This is different to the typical stimulation patterns with fibroblasts which involve plasminogen activation. Therefore, there is substantial benefit from pursuing investigation of a co-culture system. The major drawbacks of this approach are the need for both an oral mucosa and a subcutaneous skin biopsy and the time taken to culture adipose derived stem cells, which is twice as long as fibroblasts.

Further *in vitro* work should continue to assess the effect of mechanical and chemical stimulation. The proof of concept studies have shown benefit from using variable strain and Asc-2p. The application and variation of strain in our preliminary experiments were crude and a bioreactor to replicate pelvic floor strains should be explored as evidence in the literature also suggests this may stimulate fibroblasts to produce good ECM capable of accepting the applied strain [508] [592] [593]. The growth factors that have been suggested from the literature are platelet derived growth factor and basic fibroblast growth factor [594] [595], insulin like growth factor-1 [596], transforming growth factor β-1 [597] and epidermal growth factor [598], as well as vanadate [599] and oestrogen receptor β agonists[600].

The use of hormones, growth factors and drugs integrated into biodegradable scaffolds has also recently attracted interest in the field of tissue engineering [601]. Work from our own laboratory has shown that scaffolds may be mixed with ibuprofen which releases as the scaffold degrades [601]. The obvious hormone that may benefit a tissue engineering application for SUI/ POP is oestradiol as it is used as a first line treatment for extrusion of mesh in to the vagina. A slow release of oestrodiol from the scaffold may help prevent extrusion/ erosion.

The effect of the length of culture prior to implantation should also be defined to guide surgical practice. Although it is shown two weeks of culture produces good prostheses, a window needs to be defined of prosthetic availability to allow operative planning. Further

investigation of the one stage approach is also warranted as this would take a large cost away from the process and makes it accessible to surgeons geographically distant from an approved laboratory clean room. Moving on from our early findings, techniques to improve cellular outgrowth need to be investigated. These experiments are on-going in other projects of tissue engineering for skin and cornea within the laboratory at present.

Moving on to *in vivo* work, the host response to the tissue engineered prostheses needs to be investigated in animal models. The assessment of tissue integration, cell survival, vascularisation and new ECM production does not require a pathological model. The macrophage phenotype (M1 or M2) may also be investigated but at present we are unsure of its significance. In addition to this, the strength of the graft at various time points needs to be examined.

Progression to a clinical pilot study can only be achieved if it is shown that there is no adverse inflammatory response to the scaffolds which integrate well into native tissue. One needs to prove transplanted cells survive beyond the survival of the scaffold and continue to produce new collagen which strengthens the weak native tissue.

With respect to animal models, the use of pathological models has its limitations and strategies such as sciatic nerve denervation or surgical creation of structural defects do not always create a scenario akin to SUI/ POP in women. Nevertheless they may still shed some light on efficacy and failure. A pathological model may help guide how much contraction is acceptable from a tissue engineered prosthesis in a situation where it may be required. Also if excess contraction was to occur it will inform us if urinary retention or erosion occur.

Although it seems as though there is a vast amount of work still required and many avenues unexplored, the above list of suggestions for future work are by no means essential for progression to a pilot study. As shown in Fig 10.4.1 the results of the *in vivo* study will dictate progression to a clinical pilot study. The extra work highlighted and discussed above can be used to improve upon the design of prostheses even after they have reached a clinical stage thereby providing avenues for continued development and improvement in patient care.

Conclusions

- Current prostheses for SUI/ POP fail due to a poor inflammatory response, poor tissue integration and a lack of new collagen production.

- Cell seeded prostheses have the ability to overcome the deficiencies in current prostheses

- Properties desired of a tissue engineered prosthesis have been described

- Incomplete correlation between the mechanical properties of current prostheses and their success and erosion rates have been shown

- Oral fibroblasts may be a suitable cell type for a tissue engineered prosthesis for SUI/ POP

- From a comparison of seven materials, SIS and Th PLA have been identified as good scaffolds for further study based on their ability to support cells, support the production of new extracellular matrix and their mechanical properties being close to those of native tissue

- The use of simple fixed restraint did not show benefit in improving the properties of tissue engineered prostheses (except for PPL which was otherwise a poor scaffold) and in reducing *in vitro* contraction

- The addition of simple variable stress showed a small benefit in improving the properties of tissue engineered prostheses and should be investigated further

- The use of Asc-2p has been shown to improve collagen production by cells on a PLA matrix and should be investigated further particularly in combination with dynamic strain.

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Appendices

1 Data collection tables of the biomechanical testing of pelvic organ prostheses

2 Patient information leaflet for buccal mucosa samples

Appendix 1

Author	Method of testing	Sample	Ultimate tensile strength (MPa)	Ultimate strain	Young's modulus (MPa)	Notes
Hilger *et al.* 2006 [296]	Uniaxial, servohydraulic testing machine	Autologous fascia (Rabbit)	1.57	-	4.67	
		Porcine dermis	5.14	-	26.72	
	Clamp grip	Porcine collagen-coated polypropylene mesh	4.74	-	4.53	
	Baseline values	Human cadaveric dermal graft	10.68	-	30.59	
	Post 12 weeks in rabbit abdomen/vagina	Autologous fascia	1.12	-	3.3	
		Porcine dermis	0.82	-	4.78	
		Porcine collagen-coated polypropylene mesh	3.52	-	3.17	
		Human cadaveric dermal graft	1.43	-	8.26	

Author	Method of testing	Sample	Ultimate tensile strength (MPa)	% relative elongation at failure	Young's modulus (MPa)	Notes
Pierce *et al*. 2009 [297]	Uniaxial testing, 5mm/min	Porcine dermis (baseline)	2.31	54.75%	9.18	
	Rate, clamp grip, wrapped in sterile moist gauze, refrigerated and tested	Polypropylene (baseline)	2.59	40.97%	9.04	Macroporous monofilament (Gynecare)
	within 24 hours	Porcine dermis post 9 months in rabbit vagina/abdomen	1.84	35.11%	7.27	
		Polypropylene post 9 months in rabbit vagina/abdomen	1.6	3.76	87.48	

Author	Method of testing	Sample	Ultimate tensile strength (MPa)	% relative elongation at failure	Notes
Rudob *et al.* 2007 [298]	Uniaxial, 2mm/sec rate All samples underwent freeze thaw, kept in sat solution	Non prolapsed cadaveric tissue	0.82-2.62	20-46%	Data described as non- linear therefore Young's
	whilst thawing, clamp grip (PROTOCOL as per Ref 1)	Prolapsed tissue from patient	2.12-6.06	19-41%	Modulus not available

Author	Method of testing	Sample	Baseline (Kg)	6 weeks implantation in rat abdomen (kg)	12 weeks implantation in rat abdomen (Kg)	Notes
Speiss *et al*. 2004 [299]	Uniaxial, kept in N saline clamp grip, force required to rupture	TVT (polypropylene)	0.74	0.58	0.27	No account of tissue size
		Cadaveric fascia lata	0.39	0.17	0.19	No account of tissue size

Author	Method of testing	Sample	Maximum load (N)	Displacement (mm)	Notes
Choe *et al*. 2001 [300]	Uniaxial, testing machine Clamp grip Full length graft	Human fascia lata	217	1.36	No account of tissue size,
		Cadaveric freeze dried human fascia lata (Alloderm)	144	3.12	
		Polytetra fluoroethylene (Gore-Tex)	136	57.0	
		Prolene	134	28.3	
		Human dermis	122	8.83	
		Rectus fascia	42	5.87	
		Vaginal wall	42	6.25	
		Human Fascia lata	58	31.5	
	Graft attached to 0/1 prolene suture	Cadaveric freeze dried human fascia lata (Alloderm	68	27.8	
		Polytetra fluoroethylene (Gore-Tex)	76	51.8	
		Prolene	63	67.8	
		Human dermis	75	48.9	
		Rectus fascia	38	30.7	
		Vaginal wall	21	15.5	

Author	Method of testing	Sample	Low stiffness Load/elongation (N/mm)	High stiffness Load/elongation (N/mm)	Load at failure (N)	Relative elongation at failure (%)	Cyclical testing %permanent elongation	Notes
Jones *et al*.	Uniaxial, testing machine	Gynecare	0.27	1.25	68.34	71.5	20.3	No account of tissue size
2009 [301]	Clamp grip	Popmesh	N/A	0.36	21.4	60.95	32.1	
	Tested in N saline	Polyform	0.05	0.69	51.67	92.25	29.6	
	50mm/min	Pelvitex	0.07	0.87	55.35	100	39.5	
		Timesh	0.02	0.17	9.62	61.66	N/A	

Author	Method of testing	Sample	Low stiffness Load/elongation (N/mm)	High stiffness Load/elongation (N/mm)	Load at failure (N)	Relative elongation at failure (%)	Cyclical testing %permanent elongation	Notes
Moalli *et al*.	Uniaxial, testing machine	Gynecare	0.09	2.0	73.5	108	17.5%	No account of tissue size
2008 [302]	Clamp grip	Boston Scientific	0.05	1.9	69.6	107	11.9%	
	Tested in N saline	AMS	0.09	1.7	79.2	115.2	11.6%	
	50mm/min	Bard	0.16	1.2	59.8	92.3	7.7%	
		Caldera	1.2	N/A	82.0	105.9	2.5%	
		Aris	1.5	N/A	56.3	42.7	0.3%	

Author	Method of testing	Sample	Maximum load (N)	Maximum % strain	Notes
Krause *et al*.	Uniaxial, servohydraulic testing machine	Gynemesh (Monofilament polypropylene)	37.5	63.4	No account of tissue size
2008 [303]	Clamp grip	TVT (Monofilament polypropylene)	77	113.4	
	Rate- 2mm/s	Prolene (Monofilament polypropylene)	122	66.6	
		SPARC (Monofilament polypropylene)	66.8	135.3	
		Vypro (combined monofilament polypropylene & multifilament polyglactin)	100	57.2	
		Dexon (Multifilament polyglactin)	105	110	
		Vypro II (combined monofilament polypropylene & multifilament polyglactin)	24.5	81	
		Atrium (Monofilament polypropylene)	95.4	80	
		IVS (Multifilament polypropylene)	50.8	47.8	

Author	Method of testing	Sample	Maximum load/graft width (n/mm)	Ultimate strain	Stiffness (n/mm)	Notes
Lemer *et al*.	Uniaxial, tissue kept in	Autologous rectus fascia	33.2	-	113	No account of
1999 [304]	N.Saline	Solvent dehydrated fascia lata	31.9	-	114.5	tissue thickness
	Clamp grip	Freeze dried Fascia lata	25.0	-	89.2	
		Dermal graft	31.9	-	118.3	

Author	Method of testing	Sample	Maximum load (N)	Stiffness(Tangent of load/ displacement) N/mm	Notes
Dietz *et al*.	Uniaxial, tested in air	TVT (Monofilament polypropylene)	68.1	0.23	No account of
2003 [305]	Roller grip	SPARC (Monofilament polypropylene)	52.1	0.53	tissue size
	Rate- 1200mm/min	Prolene (Monofilament polypropylene)	56.4	0.53	
		Mersilene (polyester)	50.3	1.17	
		IVS (Multifilament polypropylene)	46.2	1.58	
		Gore-Tex Mycro mesh (Expanded PTFE)	71.3	2.61	
		Gore-Tex Soft tissue patch (Expanded PTFE)	84.1	2.68	
		Nylon	422	6.83	

Author	Method of testing	Sample	Low stiffness Load/elongation (N/m)X1000	High stiffness Load/elongation (N/m)X1000	Notes
Afonso *et al*.	Uniaxial, testing machine	Aris	2.39	5.37	No account of tissue size
2007 [306]	5mm/sec	TVTO	0.95	1.31	
		Uretex	1.14	3.22	
		Avaultra	1.79	3.78	
		Autosuture	1.05	2.26	

Author	Method of testing	Sample	Ultimate tensile strength (MPa)	Ultimate strain	Young's modulus (MPa)	Notes
Walter *et al*.	Uniaxial, wrapped in gauze & refrigerated until testing	Human cadaveric fascia	29.6	-	178.8	
2003 [307]				-		
		Human cadaveric fascia Post explantation at 12weeks from rabbit vagina	2.0	-	8.1	

Author	Method of testing	Sample	Breaking point in rigid orientation (N)	Breaking point in soft orientation (N)	Notes
Boukerrou *et al*.	Uniaxial, testing machine Tested until failure	Prolene	85	54	No account of tissue size
2006 [308]	Velcro grip	Prolene soft	33	31	
	Samples kept in physiological	Mersuture	42	21	
	Serum for a few	Vicryl	26	55	
	Hours	Vypro	42	20	
	10 weeks implantation in pig abdomen	Prolene	74	70	
		Prolene soft	41	35	
		Mersuture	39	37	
		Vicryl	21	15	
		Vypro	40	21	

Author	Method of testing	Sample	Ultimate tensile strength Baseline (MPa)	Ultimate tensile strength 2 weeks explantation (MPa)	Ultimate tensile strength 4 weeks explantation (MPa)	Ultimate tensile strength 8 weeks explantation (MPa)	Notes
Atmaca *et al.* **2008** [309]	Uniaxial, 1kN load cell Rabbit abdomen	Prolene	25.2	15.9	12.6	14.0	
		Mersilene	9.2	10.2	11.4	8.5	
		Ultrapro	2.2	14.5	8.9	12.5	
		Vypro	8.1	14.4	9.5	7.0	

Author	Method of testing	Sample	Ultimate strain Baseline	Ultimate strain 2 weeks explantation	Ultimate strain 4 weeks explantation	Ultimate strain 8 weeks explantation	Notes
Atmaca *et al.* **2008** [309]	Uniaxial, 1kN load cell Rabbit abdomen	Prolene	1.81	0.87	1.01	0.79	
		Mersilene	1.31	1.0	0.97	1.1	
		Ultrapro	0.91	1.08	0.91	0.90	
		Vypro	0.84	0.82	0.85	0.55	

Author	Method of testing	Sample	Max load (Kg) Baseline	Max load (Kg) 6 weeks explantation	Max load (Kg) 3 months explantation	Max load (Kg) 6 months explantation	Max load (Kg) 9 months explantation	Max load (Kg) 12 months explantation	Notes
Zorn *et al*.	Instron tensiometer Clamp grip	TVT	0.779	0.525	0.685	0.697	0.569	0.523	
2007 [310]	Tested <3hours of harvest	SPARC	0.453	0.517	0.597	0.450	0.515	0.497	
	Implantation in rat abdomen	STRATASIS	0.402	0.390	0.229	0.214	0.168	0.174	