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COLLAGEN COMPOSITION IN DIFFERENT ETHNIC GROUPS AND CHANGES IN PELVIC FLOOR DYSFUNCTION

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ABSTRACT

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COLLAGEN COMPOSITION IN VAGINAL SKIN OF DIFFERENT ETHNIC GROUPS AND CHANGES IN PELVIC FLOOR DYSFUNCTION

by Elisenda Laborda Rouch

Black women have a much lower incidence of stress incontinence (SI) and pelvic organ prolapse (POP) compared to white women. The composition of connective tissue of a black woman's vaginal skin could shed light into why a greater resistance to pathophysiological mechanisms leading to pelvic floor dysfunction (PFD) exists in black women. Secondly, identifying vaginal areas of the greatest changes in collagen metabolism could also give us further insights into finding remedies for PFD.

Two complementary studies were designed to study, in terms of molecular expression, resilience and dysfunction in the pelvic floor. The hypotheses are, firstly, that differences in collagen between black and white women will show tissue characteristics that protect black women's connective tissue from damage. Secondly, different areas of vaginal skin affected by prolapse will show changes in collagen metabolism markers which could reflect a stress related attempt at adaptation of vaginal tissues.

Paraurethral vaginal skin of matched white and black women from South Africa with and without SI was obtained and structural collagens I and III, collagen XVII mRNA expression and active matrix metalloproteinase 2 (MMP2) were analyzed. Further samples were obtained from stretched and non stretched vaginal skin of white women affected by prolapse and similarly collagen XVII mRNA expression and active MMP2 were measured as well as total MMP2.

White women with SI had significantly reduced collagen I:III ratio (an indication of increased elasticity) compared to their controls ($p<0.002$). Interestingly, black controls had a significantly lower collagen I:III ratio compared to white controls ($p<0.05$). A significant up-regulation of collagen VXII, a marker of adhesiveness, was seen in the white SI group ($p<0.02$) compared to their respective control groups. Interestingly, again following the white SI pattern, collagen XVII expression was significantly increased in the black control group compared to white controls ($p<0.05$). There were no racial differences in MMP2 activity per unit of protein (ng/mg). In vaginal prolapse, collagen XVII expression in stretched vaginal tissue was significantly lower compared to the matched non-stretched group ($p<0.02$) and a significant decrease ($p<0.02$) in the active MMP2 per unit of protein in the stretched tissue compared to non-stretched was also noted.

These findings indicate that black women have a more elastic and adhesive tissue as reflected in lower collagen I:III ratio and increased collagen XVII representing an advantage when confronted with tissue insults and protecting the tissue from damage. It appears white women with stress incontinence try to change their collagen composition to a more advantageous tissue similar to the properties of a black woman by increasing tissue elasticity and adhesiveness, but obviously not always with success.

As an insult increases as in vaginal prolapse, increased active MMP2 expression occurs but in the more stretched vaginal areas adhesiveness becomes lost (lower collagen XVII) and also some tissue remodelling.

Finding ways to increase elasticity and adhesiveness of vaginal skin further may provide remedies for preventing or alleviating PFD.

List of contents

List of figures

INTRODUCTION

Chapter 1: Introduction

METHODOLOGY

Chapter 2: Tissue sampling

Chapter 3: Total protein extraction

Chapter 4: Enzyme immunoassay technique

Chapter 5: Molecular biology

Chapter 6: Immunohistochemistry

RESULTS

Chapter 7: Enzyme immunoassay technique

Chapter 8: Molecular biology

Chapter 9: Immunohistochemstry

DISCUSSION

Chapter 10: Discussion

List of Tables

INTRODUCTION

Chapter 1: Introduction

METHODOLOGY

Chapter 2: Tissue sampling

Chapter 3: Total protein extraction

Chapter 4: Enzyme immunoassay technique

Chapter 5: Molecular biology

RESULTS

Chapter 7: Enzyme immunoassay techniques

Chapter 8: Molecular biology

Chapter 9: Immunohistochemistry

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Abbreviations

ACE	3-amino 9- ethyl carbazole
ADAM	A desintegrin and metalloproteinase
APES	Aminopropyltriethoxysilane
APMA	Aminophenylmercuric acetate
BC	Black control
BMI	Body mass index
BSA	Bovine serum albumin
BSI	Black stress incontinence
°C	Centigrade
cDNA	Complement Deoxyribonucleic Acid
Ct	Cycle threshold
CV	Coefficient of variance
DAB	Diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic Acid
DNAases	Deoxyribonucleases
DMSO	Dimethylsulphoxide
ELISA	Enzyme –linked immunosorbent assay
g	Gram
GTC	Guanidine thiocynate
HRT	Hormone replacement therapy
5-HT	Serotonin
ICS	International Continence Society
KDa	KiloDalton

mg	Miligram
min	Minutes
ml	Millilitre
MMP	Matrix metalloproteinase
MRI	Magnetic resonance image
mRNA	Messenger Ribonucleic Acid
NA	Noradrenalin
ng	Nanogram
nm	Nanometer
NTC	Negative control
PBS	Phosphate buffered saline
POP-Q	Pelvic organ prolapse questionnaire
RNA	Ribonucleic acid
RNAase	Ribonuclease
RT-PCR	Reverse transcription protein chain reaction
Rpm	Revolution per minute
Sec	Seconds
Sem	Standard error of the mean
SI	Stress incontinence
SIS	Small intestinal submucosa
SUI	Stress urinary incontinence
TIMP	Tissue inhibitor matrix metalloproteinase
TOT	Transobturator tape
TVT	Tension free vaginal tape

WC	White control
WSI	White stress incontinence
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromol

Presentation of work related to thesis

Free communications:

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INTRODUCTION

Chapter 1:

Background

Background

1.1 Pelvic floor dysfunction

Pelvic organ prolapse and urinary stress incontinence are forms of pelvic floor dysfunction. They share similar etiological factors and therefore it is not uncommon they coexist in the same woman [1]. Despite significant advances in treatment and surgical techniques, the exact pathophysiology and underlying mechanisms resulting in these conditions is still not well understood. This makes the prevention difficult. There are several factors which are known to contribute to the development of these conditions, particularly pregnancy, childbirth and menopause [2]. The role of connective tissue in pelvic floor disorders is increasingly implicated as research in this field advances. More recently it has been suggested that genetic influences, which determine connective tissue make up, could play an important role in predisposing a woman to pelvic floor dysfunction (PFD). A high concordance of pelvic organ prolapse in nulliparous and parous sister pairs suggests a familial predisposition towards developing this condition [3]. Establishing genetic markers to identify women at risk or developing new therapies to influence the characteristic of the tissues of the pelvic floor to treat these conditions would mean a real advance in the treatment of pelvic floor disorders.

My research work is orientated to contribute to a better understanding of the mechanisms that lead to pelvic organ dysfunction and to set the basis for the future development of tissue engineering techniques in conjunction with the possibility of administrating hormones and cytokines to achieve certain tissue properties for the treatment of stress incontinence (SI) and vaginal wall prolapse.

1.1.1 Epidemiology of pelvic floor dysfunction

Epidemiological studies try to identify the frequency and distributions of diseases in a community. Studies of pelvic organ disorders are difficult to perform accurately. We understand by pelvic organ disorders a group of conditions including urinary incontinence and pelvic organ prolapse. The definition of urinary incontinence has been changed by investigators several times and incontinence is a condition which includes many different types. Stress urinary incontinence is a very frequent condition affecting 40 to 60% of women at different stages of life. Different surveys of women visiting their GP have revealed that 47% of women were incontinent in USA [4] and 44% in Sweden [5]. Despite the fact that stress urinary incontinence is more frequent in later life five percent of young nulliparous women will suffer from stress urinary incontinence. Twenty five percent of young female athletes have been reported to suffer from stress urinary incontinence [6]. However stress urinary incontinence is still highly associated with childbirth and menopause.

The true incidence of pelvic organ prolapse is also very difficult to assess due to the numerous asymptomatic women suffering from pelvic floor defects. It is estimated that the life time risk for surgery for prolapse or stress incontinence is 11% and one in three

women will require more than one surgical repair [7]. An Australian epidemiological study has shown that 46 % of women between 15 and 97 had symptoms of pelvic floor dysfunction which have a strong association with female gender, ageing, pregnancy, parity and instrumental delivery [8], which are all well recognized risk factors for pelvic organ prolapse together with alteration in connective tissue structure and its metabolism.

The incidence of pelvic organ prolapse is also race dependent. The prevalence of urinary incontinence was found to be higher in Hispanic women living in America, followed by white women then black and Asian -Americans[9]. In a previous study white, Asian and Hispanic women were found to have a similar prevalence of stress urinary incontinence and African-American had lower rates compared to them [10]. Another American study including 183 African-American women and 132 Caucasians with symptoms of urinary incontinence and prolapse found that the prevalence of urinary incontinence in the African-American group was significantly lower compared to the Caucasian one (22% vs 46%) [11].

1.1.2 Classification of pelvic floor dysfunction

There are two main groups of pelvic floor dysfunction. The first group includes disorders of the lower urinary tract and the second one includes pelvic organ prolapse. They both have similar pathophysiology and can often present concurrently. There is no doubt that a relationship exists between childbirth and damage to the pelvic floor, which is one of the main pathophysiological factors resulting in pelvic floor dysfunction. However this does not explain why nulliparous women and only some of the parous

women will develop incontinence and prolapse. Several studies have focused on the possibility of a genetic component which would predispose a woman to develop pelvic floor dysfunction.

Stress urinary incontinence

The type of incontinence that is mostly associated with alterations of the pelvic floor tissue is stress urinary incontinence. This is the involuntary leakage associated with an increase in intraabdominal pressure. The diagnosis is made by accurate history taking, thorough physical examination and confirmed by urodynamic studies when leakage is confirmed in the absence of detrusor contraction.

Stress incontinence can be due to an intrinsic sphincter deficiency or due to a hypermobile urethra secondary to a decrease in tissue support. The existence of pure cases is rare and the majority of cases have both mechanisms involved in their pathogenesis. One of the favoured classifications of stress incontinence was devised by Green in 1962 where he divided it in to three groups as shown in table 1.1.

Type I	Absence of the posterior urethrovesical angle without urethra hypermobility
Type II	Absence of the posterior urethrovesical angle with urethra hypermobility
Type III	Fixed urethra with low urethral closure pressure

Table 1.1 Classification of stress urinary incontinence

More recently Wein has introduced a classification, which differentiates voiding problem from storage problems, and each group was therefore subdivided in to outlet or bladder problems.

Pelvic organ prolapse

Pelvic organ prolapse is defined as the protusion of a pelvic organ beyond its anatomical boundaries. Pelvic organ prolapse is divided into three different compartments depending on the organ involved (table 1.3).

Anterior compartment	Urethrocele
	Cystocele
Posterior compartment	Enterocele
	Rectocele
Apical compartment	Utero-vaginal prolapse
	Apical prolapse

Table 1.2 Classification of pelvic organ prolapse

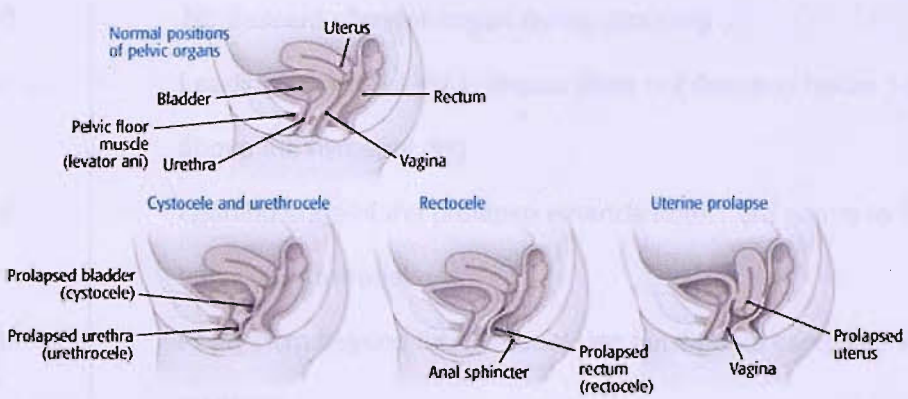


Figure 1.1 Anatomical representations of different types of pelvic organ prolapse

The severity of pelvic organ prolapse is graded by different methods. Clinically the most used method is the Baden & Walker classification which recognizes four different grades (table 1.3)

Grade I	Descent of any organ to the vaginal midplane
Grade II	Descent to the hymenal ring
Grade III	Descent to halfway through the introitus
Grade IV	Complete eversion

Table 1.3 Classification of pelvic organ prolapse severity.

The POP-Q is an objective method standardized by the International Continence Society Committee [12] . As part of the classification it recognizes five different stages as described in table 1.4.

0	No descent of pelvic organ during straining
I	Leading surface of the prolapse does not descend below 1 cm above the hymenal ring
II	Leading edge of the prolapse extends from 1 cm above to 1 cm below the hymenal ring
III	From 1 cm beyond the hymenal ring but without complete vaginal eversion
IV	The vaginal is completely everted

Table 1.4 POP-Q classification

1.1.3 Anatomy and support of female pelvic cavity

In a woman, the urinary system is in close relationship to the reproductive system, the gastrointestinal tract as well as the pelvic floor. It is important to appreciate this relationship and the anatomy of each of the systems to have a comprehensive understanding of the continence mechanisms in women and why incontinence can occur.

The bladder, which lies behind the pubic bone, is a muscular organ whose wall contains three layers of non striated muscle or detrusor, inner and outer longitudinal layers and a middle circular layer. The bladder is covered by a serosa and adventitia layer. There is only peritoneum at the fundus. The inner layers consist of a transitional epithelium and submucosa layer. Some fibres of the detrusor muscle on the anterior aspect of the bladder insert in the pubic bone and pelvic side walls creating the

pubovesical muscles. The base of the bladder or trigone is separated from the vaginal wall by pubocervical fascia. This fascia is the net which surrounds the pelvic organs and is responsible in part for their support.

The pelvic fascia is divided in to parietal fascia and visceral fascia. Over expansible parts the fascia is also expansible and lacks a membrane. The fascia is a strong membrane over non expansible structures, like the fascia of the pelvic wall covering the obturator internus and piriformis muscles and strongly attached to the periostium. The fascia of the pelvic floor is strongest at the insertion of the muscles into the pelvic wall and when it condenses to form ligaments for the suspension of the pelvic organs. Nevertheless, the fascia which fills the spaces between bladder, vagina and rectum is distensible and composed of loose areolar tissue which allows compression during pregnancy and childbirth.

The urethra lies on a layer of endopelvic fascia and anterior vaginal wall. The fascia is different at different levels, at the bladder neck it is minimal and as the urethra approaches the introitus the quantity and density increases. The urethra is surrounded by endopelvic fascia which supports it in conjunction with the anterior vaginal wall. Laterally the fascia attaches to the pelvic side wall through the arcus tendineous fascia which is suspended from the pubic bone, where it becomes a strong and well defined tendon, to the ischial spines. Posteriorly, the arcus tendineous fuses to the aponeurosis of the levator ani muscle which extends medially to the vagina wall as a broad aponeurotic sheet suspending the pelvic structures.

In young women a clear differentiation between the endopelvic fascia, vaginal muscularis and vaginal submucosa exists. In older age this differentiation disappears and these structures become fused as the endopelvic fascia becomes thinner [13].

Surrounded by fascia there are a group of muscles forming a double layer sheet in a U shape which keep the intraabdominal organs (bladder, uterus, vagina, bowel and rectum) suspended in place. In the inner layer, the largest muscle is the levator ani which consists of the pubococcygeus which arises from the anterior half of the white line and from the posterior surface of the body of the pubis on the level of the lower border of the pubic symphysis and lateral to it there is the iliococcygeus, which arises from the posterior half of the white line and the pelvis surface to the ischial spine.

The urethra goes from the bladder neck to the external urethral meatus running anteroinferiorly behind the synphisis pubis. It is immediately related to the anterior vaginal wall inferiorly and laterally to the urogenital diaphragm and the levator ani (pubococcygeus and iliococcygeus). The vagina and the endopelvic fascia support the urethra and bladder neck. The fascia extends towards the arcus tendineous fascia pelvis and levator ani muscle where it gains support creating a hammock on which the urethra lies. When the intraabdominal pressure rises the urethra is pushed against it compressing and closing the lumen and therefore maintaining continence [13]. This is one of the mechanisms which contributes to urinary continence.

The vagina is in close contact posteriorly with small bowel, pelvic colon and upper rectum which is separated from it by pelvic fascia. The vagina is supported laterally by the broad ligaments and at the mid-portion by the pubocervical and rectovaginal fascia.

At the lower third it is in close contact with the perineal body. Weakness in vaginal support can originate cystocele and rectocele.

Fascia, muscle and ligaments are important for urethral support and therefore continence as well as vaginal support. When this support fails, in the event of a rise in intraabdominal pressure stress incontinence can occur. Pelvic organ prolapse will develop as a result of weak tissues.

1.1.4 Continence mechanisms

There are different theories regarding the continence mechanism in women and it seems that all of them agree that multiple factors are involved. There is not one unique mechanism responsible for urinary continence.

The intraabdominal position of the bladder neck and its slight posterior angle of the bladder neck created by the levator ani ensure that when a rise in intraabdominal pressure occurs it is also transmitted to the bladder neck, thereby maintaining the pressure gradient. This mechanism is enhanced by the underlying structures which supports the urethra. The continuum of the anterior vaginal wall, levator ani and arcus tendineous by the endopelvic fascia and the paravaginal tissue helps the urethra to compress when a rise in intraabdominal pressure takes place.

The pubococcygeus muscle contraction causes urethral closure as well as the extrinsic urethral sphincter fibres. Petros and Ulmsten's Integral theory of female urinary incontinence describe the pubococcygeus muscle as the first level of the closure

mechanism that achieves continence. The contraction of the anterior pubococcygeus muscle causes the vaginal hammock to swing forward and therefore the urethra closes. The second level, at the bladder neck, requires intact uterosacral and pubourethral ligaments which support and fix the proximal urethra. Contraction of the pubococcygeus muscle produces an elongation backwards and downwards of the bladder neck when the proximal urethra is fixed by good ligamentous support. The distribution of the muscular fibres at the urethrovesical junction forms the urethral internal sphincter where they are mainly circular and are responsible for continence. The external sphincter is formed by voluntary muscle fibres surrounding the urethra. Distally some fibres attach to the pubic bone forming the compressor urethrae and the vagina forming the urethrovaginal sphincter. Beneath the striated muscle there is a layer of smooth muscle, circular and longitudinal, which is responsible for the constriction of the lumen and the shortening and funnelling of the urethra during micturition. The striated fibres are involuntary muscle fibres and are slow twitch helping maintain a constant tone responsible for urethral resistance and closure (resting urethral closure pressure) except during micturition.

The third mechanism of urethral closure is at the level of the puborectalis and pubococcygeus which if trained can be involved in urethral closure [14]. Several studies have demonstrated that pelvic floor retraining alone is an effective treatment for urinary stress incontinence and is superior to other conservative treatments like electrical stimulation or the use of vaginal cones [15].

The first continence mechanism relies on a good isometric muscle contraction, which requires strong muscle insertions and therefore connective tissue. The second mechanism is based on the support given by ligaments and fascia.

Taking this into consideration we could say that connective tissue is a crucial constituent of the pelvic floor in maintaining continence and structural support to the pelvic organs, in conjunction with the pelvic floor muscles. We decided to study pelvic fascia for a number of reasons. Skeletal muscle is more sensitive than tendon to mechanical stimulus, seen by a more marked increase in the expression of TGF-beta 1, collagen I and III in response to muscle contraction in rat skeletal muscle compared to tendon [16]. This could indicate that physical activity could have a lesser impact in connective tissue and therefore pelvic fascia. Physical activity is a factor which would be very difficult to control for in a study design like this one. Taking this into consideration and the important role that fascia has in pelvic floor function and tissue accessibility we felt it was more appropriate to study vaginal skin as representation of pelvic fascia. The correlation between endopelvic fascia and vaginal skin constituents has been previously described.

Possible changes in not only connective tissue composition but also in its metabolism between black and white African women could explain the difference in stress incontinence and prolapse incidence between black and white women.

1.1.5 Pathophysiology of pelvic floor dysfunction

The pathophysiology of pelvic floor dysfunction remains unclear. It is probably multifactorial where an accumulation of different risks factors will increase the likelihood of a woman developing pelvic floor dysfunction. There is a known association with pregnancy, childbirth, menopause and connective tissue disorders. Chronic increase in intra-abdominal pressure in situations like chronic constipation, repeated lifting or obesity, neurological conditions, smoking, deficiency of vitamin C and race are other contributing factors. All these risk factors have a direct or indirect impact in the connective tissue properties which maintains the structure and function of the pelvic floor.

Damage to the pelvic floor connective tissue would compromise the support that it provides to the intraabdominal organs. Vaginal delivery is one of the most obvious causes of pelvic floor damage but the role of permanent chronic insults to the tissues and ligaments should not be underestimated. Ageing and menopause play important roles and their effects are probably mediated through loss of tissue strength and elasticity resulting in compromised organ support.

This influence of each of these risk factors to the pelvic floor has been analyzed independently.

Pregnancy

Pregnancy changes connective tissue properties increasing the laxity and increasing collagen degradation and remodelling. Rectus fascia from pregnant women has lower collagen content and increased glycosaminoglycans and it seems to have a reduced tensile strength compared to the rectus fascia of non pregnant women having an abdominal operation [17]. Bladder and urethral mobility increase in pregnancy, as well as elbow hyperextension, as early as 6-18 weeks gestation [18]. Cervical ripening has been associated with cervical increased proteoglycan to collagen ratio, increased hyaluronic acid and water content and breakdown of collagen by matrix metalloproteinases. Collagen I is decreased in the cervix of pregnant women in the third trimester compared to the first trimester, which could play a role in the maintenance of pregnancy [19]. All these changes may have a permanent effect on connective tissue after pregnancy. Studies with longer follow up, analyzing collagen properties before, during and long after pregnancy are needed to establish the real effect of pregnancy and delivery on a woman's connective tissue. Nevertheless, they are difficult to perform due to their complexity and to ethical issues.

Childbirth

Parity is one of the most important risk factors in fertile, peri and early postmenopausal ages for developing pelvic organ prolapse and stress urinary incontinence [20]. These effects seem to disappear in older age. It is then when the prevalence of pelvic floor dysfunction seems to be more related to menopausal changes and age.

Several studies have tried to establish the risk factors for pelvic floor damage after childbirth. Increased birth weight, forceps delivery and perineal trauma seem to

increase the risk of pelvic organ dysfunction postpartum while vacuum delivery and the length of second stage seems not to increase its incidence.

Childbirth has an impact on the innervations of the pelvic floor muscles which can be related to stress incontinence and pelvic organ prolapse. Women with stress urinary incontinence, with or without genitourinary prolapse have a significant partial denervation of the pelvic floor [21]. Pudendal nerve terminal motor latency is prolonged in women after vaginal delivery and seems to be longer in multiparous women compared to primiparous. Forceps delivery is associated with even longer latencies in primiparous but did not have a significant effect in multiparous women. Improvement, with signs of reinnervation, has been seen 2 months after delivery although it is less noticeable in the multiparous group. Perineal descent occurs in all women who deliver vaginally and persists after delivery. Caesarean section is protective for both, pudendal nerve terminal motor latency and perineal descent. Overall the main risk factors leading to pudendal nerve damage are multiparity, forceps delivery, increased duration of active second stage, third degree perineal tear and high birth weight [22]. Allen et al in their study showed that forceps delivery and perineal tears do not affect the degree of nerve damage [23]. A further 5 years follow up showed that persistent pudendal nerve dysfunction causes weakness in the perineal musculature as demonstrated by anal manometry [24]. A more recent study found that the decreased anal pressures after vaginal delivery normalizes in a short time while the alteration of innervation of the upper anal sphincter lasted for a long period of time speculating the relationship with the development of faecal incontinence later in life. In a complex study using 3D computer model, Lien et al showed that during the second stage of labour the nerves innervating the anal sphincter are stretched beyond the 15% strain threshold known to

cause permanent damage in appendicular peripheral nerves [25]. The degree of perineal descent seems to influence pudendal nerve strain.

In addition to nerve damage, muscle injury also occurs during childbirth. Pelvic floor muscle strength is found to be reduced immediately after vaginal delivery but it is corrected to antepartum values by 2 months. A cadaveric study showed women who have had at least one vaginal delivery in the past, compared to nulliparous, presented with myogenic changes in the levator ani muscle. These changes are found in nulliparous women with increasing age while no further increase in changes are seen in the parous women as they aged. It is well known that women with anal sphincter injuries secondary to labour are more at risk of developing incontinence and pelvic floor prolapse although the pathogenesis is not completely clear. It could be due to the weaker scar tissue left by the injury, an associated nerve injury or permanent alterations in connective tissue. A study using MRI images to analyze the pelvic floor of women with vaginal prolapse showed a 2.5 times increased likelihood to have major levator ani muscle loss, which occurs as a result of vaginal birth in women suffering from vaginal prolapse compared to the controls [26]. Women who had episiotomies during vaginal delivery seem to complain more of dyspareunia post delivery than women who delivered vaginally without episiotomy.

Several studies have focused on detecting women at risk of developing pelvic floor dysfunction. King et al (1998) showed that women with postpartum stress urinary incontinence have significantly greater bladder neck mobility antenatally than those women continent after delivery [27]. More recently, Dietz et al studied pelvic organ mobility antenatally and found that women with little pelvic organ mobility antepartum

on translabial ultrasound seem to be those with most marked delivery related changes in all parameters use to describe pelvic organ mobility in all three compartments [18;28]. They are also more likely to need a caesarean section in second stage of labour [29]. Parous twins were compared in an identical twin study and it was found that caesarean section had a 3 fold reduction in SUI risk relative to their siblings that delivered vaginally. In a study where 200 women were followed up between 2 to 5 months postpartum it was found that those who delivered vaginally, with forceps causing the most marked changes, have higher increased pelvic organ mobility at Valsalva than those who have any type of caesarean section [30]. Nevertheless another study suggested that caesarean section during active labour and vaginal delivery have similar effect on the maternal pelvic support 6 weeks postpartum [31].

Menopause

Estrogens receptors are present in the genito- urinary tract of women. Vaginal epithelium is where the highest concentration of estrogen receptors is found, however they are highly expressed in cervix, uterus, fallopian tubes, bladder and urethra. In a study examining levator ani muscle there was no expression of the estrogen receptor in the muscle fibres but it was found in levator ani fascia. Progesterone and androgen receptors are present in both tissues [32]. Estrogens have a profound influence in maintaining adequate structural integrity, explaining why menopause has such a detrimental effect on skin and connective tissue and this exacerbates the effects of aging. During menopause there is a 30% loss of skin collagen and it becomes thinner due to a probable reduction in glycosaminoglycans. Estrogen treatment seems to reverse both effects [33]. There are several studies analyzing different routes for HRT and its effect on the skin. Varila et al [34] showed an increase in hydroxyproline in

postmenopausal women after 3 months of topical estrogen cream. Rauramo et al [35] showed an increase in skin thickness of the epidermis after 3 months of oral estrogen. Holland et al [36] could find no differences in collagen content in vaginal skin after 1 year of using estrogens implants. Haapasaari et al, [37] after 1 year on oral hormonal replacement therapy found no difference in skin thickness, amount of hydroxyproline, histological changes and in treatment with estrogens only an increased in MMP2 was observed. Jackson et al [38] also found an increase in MMP2 after 6 months of oral estrogens, with an increase in immature cross-linking which would be indicative of an increase in degradation and replacement of aged for newly synthesized collagen. That could increase the strength of the tissue despite the overall decrease in collagen content. Falconer et al [39] also defended the idea of an increased turn-over in skin of postmenopausal women on HRT.

Estrogen treatment has a beneficial effect on wound healing. It has been shown to reduce wound size after 7 days compared to placebo, increase the collagen and fibronectin levels and enhance the strength of the wound at day 80. Estrogens inhibit the local inflammatory response by down regulating the macrophage migration inhibitory factor which is a pro-inflammatory cytokine involved in hormonal regulation of inflammation [40]. The delayed response in wound healing seen in the elderly can be reduced by topical estrogen in both male and female [41].

Connective tissue disorders

Other recognized risk factors for vaginal prolapse and stress urinary incontinence are connective tissue disorders. Joint hypermobility and certain genetic connective tissue

abnormalities such as Marfan's and Ehlers Danlos syndromes are associated with increased prevalence of pelvic organ prolapse and SUI [42].

Race

Epidemiological studies have shown that Black women have a lower prevalence of stress incontinence and prolapse despite similar parity and other risks factors [9]. Postnatal stress urinary incontinence has also been found significantly increased in white Caucasian women when compared to a matched black population [43]

Several other studies analyzing connective tissue disorders and body tissue composition have shown differences between black and white races. Preliminary histological studies have shown a greater collagen content in the ligaments of black South African women with prolapse compared with whites from the same region [44] . Black people have a higher bone mass and increased muscle mass, partly responsible for their lower incidence of osteoporosis and fracture of the skeleton [45] . Black South African middle to long distance runners and Black African athletes appear to have a much higher performance compared to white South African middle to long distance runners. Interestingly, several studies have attributed it to differences in muscle metabolism rather than the higher concentration of type I fibres in the muscle of black women. Different metabolic parameters such as plasma lactate accumulation rate after exercise was lower in black athletes, they had a higher time to fatigue and citrate synthase activity was also significantly higher [46 47] .

Black women have a lower incidence of pelvic organ prolapse, nevertheless they are more likely to have detrusor instability and keloid formation in wound repair [48]. Other

studies have focused their attention on the differences in the structure and function of the stress urinary continence mechanism. Howard et al [49] studied 18 black women and 17 white women, nulliparous and continent, and found that black women had significantly greater urethral volume measured by MRI and higher mean Kegel urethral closure pressure. Knobel [50] compared black women with Indian women and found that the bladder neck in black women is situated at a higher level and the urethra is longer. The primary retaining forces are superior because of better inherent urethral tone and support. Part of this support is provided by the endopelvic fascia. As DeLancey [51] proposes in the hammock hypothesis, the effectiveness of this urethral compression mechanism would seem to depend on the stability of the suburethral layer. If the suburethral layer is lax and moveable, compression would not be as effective and the transmission of abdominal pressure to the urethra would be lessened.

To date there have been no published analyses of connective tissue metabolism in women with and without stress incontinence from different races.

Genetic factors

Several studies have suggested the possibility of a genetic component which could predispose a woman to develop pelvic organ dysfunction. An epidemiological study from Norway showed that daughters of incontinent mothers (urge incontinence was not significant) had 1.3 fold risk of being incontinent. Granddaughters had a 2.4 fold increased risk of incontinence if both mother and grandmother had urinary incontinence. Female siblings had a 1.6 fold increased risk of urinary incontinence if their older sisters were incontinent [52]. A genetic contribution in bladder neck mobility

was suggested in a twin study where the correlation between bladder neck mobility was higher in the monozygotic group compared to dizygotic twins. [53]

1.2 Composition and metabolism of connective tissue

1.2.1 Composition of connective tissue

Surrounding the muscles and the nerves of the pelvic floor is the pelvic fascia composed of connective tissue. As its name indicates, this connects all the structures together allowing the pelvic floor to function as a single unit.

Connective tissue takes the stress of movement and maintains shape. It is composed of soluble polymers (proteoglycans and glycoproteins) and insoluble fibres. The main fibres are collagen and elastin. Other molecules present in the extracellular matrix are cytokines, growth factors and cell adhesion molecules (transmembrane proteins). Release of soluble cytokines and growth factors may change their biological activities and shedding the receptor may modulate their ligand binding.

Collagen is the most common protein in the body accounting for the 30% of the total protein. All different types of collagen have in common the triple helix structure, which comprises three polypeptide (alpha) chains, each with a left-handed helical configuration, wound round each other to form a right-handed super helix. Glycine occupies every third residue which is indispensable due to it being the only one small enough to fit into the centre of the helix. From the rest of amino acids forming the chain, 20% is proline and 22% hydroxyproline. The hydroxyl group is essential to

stabilize the triple helix. Lysine and hydroxylisine residues are important for the formation of stable covalent cross linking of the different collagen molecules, which are crucial in providing tissue strength.

Collagen is synthesised by fibroblasts in a pro-form, procollagen, which is secreted into the extracellular matrix from the endoplasmic reticulum via Golgi vesicles. Once in the extracellular matrix procollagen experiences some cleavage in either ends of the chain to become collagen. Then fibronectin and other proteoglycans adhere to the collagen forming tropocollagen and different molecules of tropocollagen joined end to end will form fibrils.

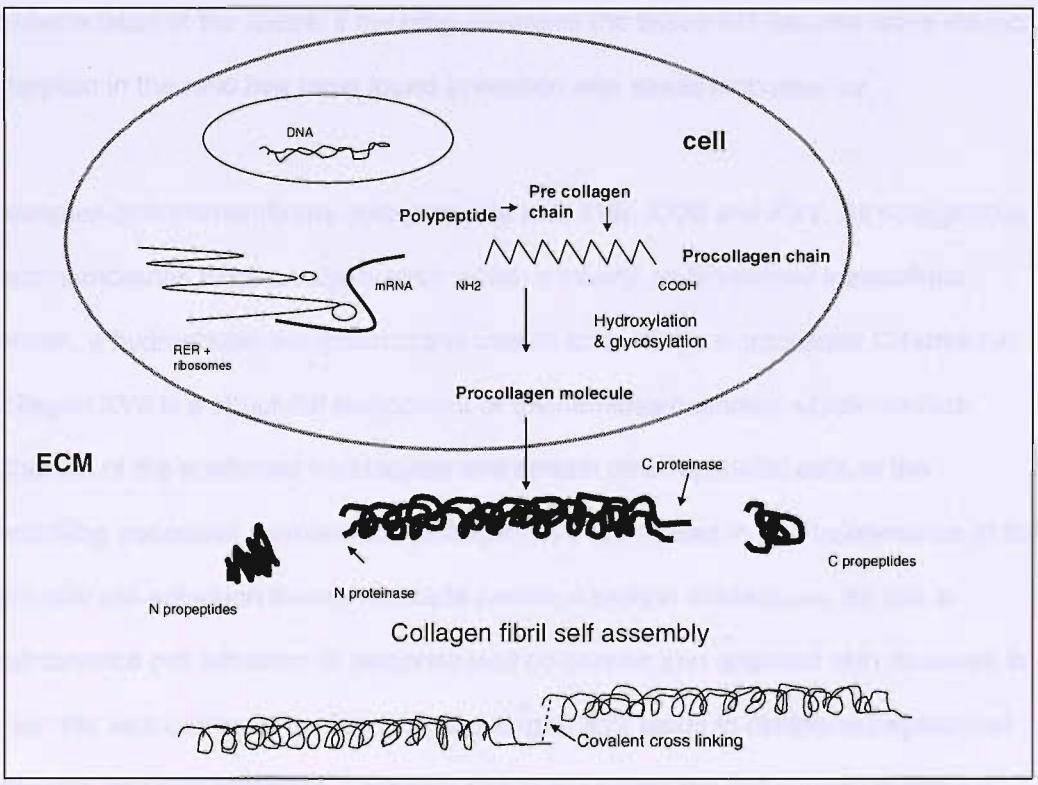


Figure 1.2 Synthesis and secretion of collagen molecule.

In skin 80% of the collagen is type I, which is a rigid fibrillar collagen and is responsible for the tensile strength of the tissue. It is composed by two alpha-1 chain disulphide bonded to one alpha-2 chain. It is present in bone, tendon and skin, here it forms sheet like structures. Alterations in its gene result in various forms of osteogenesis imperfecta and Ehlers-Danlos syndrome type VII which is characterized by joint hypermobility, which at the same time is associated with an increased risk of developing pelvic organ prolapse [42]. Fifteen per cent of the collagen in the skin is type III which is elastic. It is composed by three identical alpha-1 chains. These fibres are thin and result in a more compliant tissue. It is present in skin and vascular tissues. It is normally present with collagen type I in different proportions. Staining methods for collagen I and III are presented in chapter 6. The ratio between collagen I and III determines the characteristics of the tissue; if the ratio increases the tissue will become more elastic. Alteration in the ratio has been found in women with stress incontinence.

Examples of transmembrane collagens are XIII, XVII, XXIII and XXV. All collagenous transmembranes have an alpha-chain which contains an N-terminal intracellular domain, a hydrophobic transmembrane stretch and a large extracellular C-terminus. Collagen XVII is a structural component of the hemidesmosomes which mediate adhesion of the epidermal keratocytes and certain other epithelial cells to the underlying basement membranes. Collagen XVII is involved in the maintenance of the epithelial cell adhesion through multiple protein – protein interactions. Its role in maintenance cell adhesion is demonstrated by genetic and acquired skin diseases in which the lack or loss of the function of collagen XVII leads to diminished epidermal adhesion and skin blistering. Collagen XVII is shed from the cell surface yielding a shorter soluble form of the molecule by proteinases of the ADAMs family (ADAM-9 and

ADAM-10). The functional consequences of shedding of collagen XVII are not yet clear [54;55].

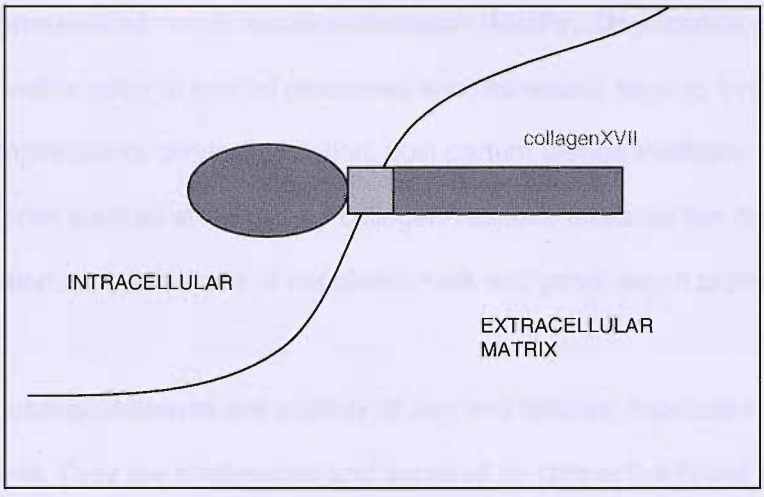


Figure 1.3 Representation of the full length collagen XVII molecule

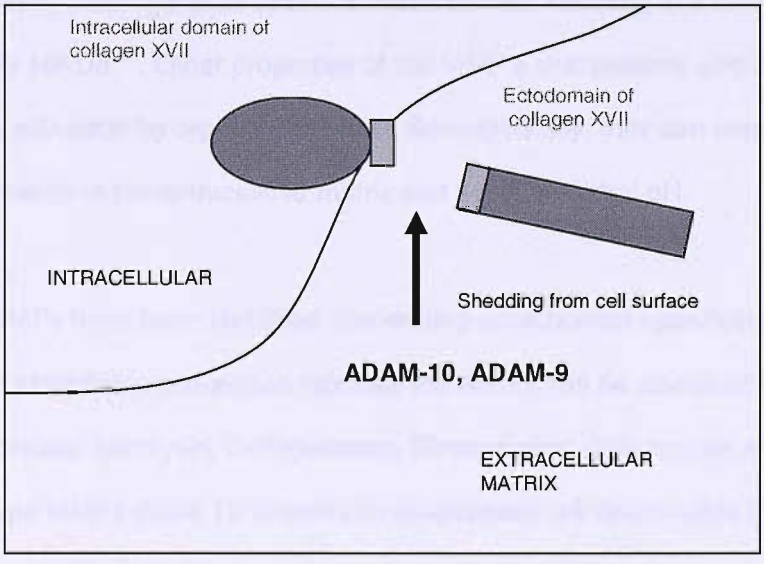


Figure 1.4 The ectodomain of collagen XVII is cleaved from the cell surface by the enzymes of the desintegrin-metalloproteinase family (ADAMs) and is released to the extracellular space.

1.2.2 Regulation of connective tissue

Collagen remodelling and degradation are tightly regulated at the molecular level by a group of enzymes called matrix metalloproteinases (MMPs). Degradation of the extracellular matrix occur in normal processes such as wound healing, ovulation, trophoblast implantation, cervical dilatation, post partum uterine involution and pathological ones such as inflammatory collagen-vascular diseases like rheumatoid arthritis, invasion and metastasis of neoplastic cells and pelvic organ prolapse [56] .

The matrix metalloproteinases are a family of zinc and calcium dependent endopeptidases. They are synthesized and secreted by connective tissue as pro-enzymes (zymogen form) which are activated through proteolytic enzymes, mercurial agents or heat by cleavage of an amino terminal domain resulting in a loss of approximately 10KDa [57]. Other properties of the MMP s are: putative zinc binding at the catalytic site, activation by organomercurials. Synergistically, they can degrade the major components of the extracellular matrix and act in a neutral pH.

At least 28 MMPs have been identified. Depending on substrate specificity, amino acid similarity and identifiable sequences modules the MMPs can be classified into five distinct subclasses: Matrilysin, Collagenases, Stromelysins, Gelatinases and Membrane type MMPs (table 1). Interstitial collagenases are responsible for fibrillar collagen degradation (type I and III). They cleave fibrillar collagen into two smaller fragments which are degraded then by gelatinases, a subgroup of MMPs. There are two types of gelatinases, A and B (MMP2 and MMP9 respectively).

SUBCLASSE OF MMP	MMP	MATRIX SUBSTANCE
Gelatinases	MMP-2	Peptides from denaturated collagen
	MMP-9	Gelatins, Elastin
Collagenases	MMP-1	Fibrillar collagen types (I, II, III, VII, VIII, X)
	MMP-8	
	MMP-13	Proteoglycan core protein
Stromelysins	MMP-3	Proteoglycan core protein
	MMP-10	Type IX and X collagen, laminin
	MMP-11	Elastin, fibronectin, Procollagens I, II
Matrilysin	MMP-7	As for stromelysins
Membrane type MMPs	MMP-12	
	MMP-15	
	MMP-16	
	MMP-14	Pro-MMP-2

Table 1.5 Matrix metalloproteinases enzymes

MMPs are proteins with 4 domain structures: pre-domain or signal structure, pro-domain which maintains the latency of the proteins, catalytic domain and C-terminal domain implicated in macromolecular substrate recognition. The pro-domain consists of an independent folding unit which binds at the active site of the catalytic region. The catalytic domain contains two Zinc and several calcium atoms. The C-terminal domain

is involved in the recognition of macromolecular substrates and has been implicated in determining the distinct substrate specificities of the various MMPs [58].

The regulation of metalloproteinases occurs at the level of gene expression and also at an extracellular level by the action of tissue inhibitors of metalloproteinases (TIMPs), which inhibit active MMPs, pro MMP activation, cell growth promotion, angiogenesis and induce apoptosis [59]. TIMPS inhibit MMP activities through non-covalent binding of the active form of the MMPs. There are 4 different types of TIMPs. They bind to pro-MMP2 and pro-MMP9 with a high degree of specificity, providing an extra level of regulation by preventing activation. TIMP1 and 2 inhibit the activity of all active MMPs. TIMP-1 is the major form and it is produced by connective tissue cells as well as macrophages and it is present in alpha granules of platelets. Its inhibitory function is through high affinity, reversible non covalent binding to form 1:1 complexes. TIMP-1 is not cleaved and it can be recovered with full activity from complexes with MMP-3. Complex formation is slow and both active collagenases and stromelysin have been found in association to alpha- 2- macroglobulin than to TIMP. TIMP-2 is less abundant but has been extracted from several tissue extracts. It has similar properties than TIMP-1 in terms of binding activity, with the exception that TIMP-2 binds the pro form of the gelatinases. TIMP-3 is found is association with the basement membrane, but is less well characterized and TIMP-4 is the most recently described and therefore the less well investigated.

A number of studies have reported that changes in TIMP-1 production are associated with events happening in the tissue. Both collagenases and TIMP could be identified in

hypertrophic scar tissue undergoing active remodelling, but were not found in quiescent tissue. [60]

Extracellular matrix homeostasis during physiological processes, such as embryonic development and ovulation, depends on the coordinated balance between deposition and removal of connective tissue matrix components. Changes in the balance between matrix degrading proteinases and their inhibitors can have profound effects on the composition of the extracellular matrix and affect various cellular functions including adhesion, migration and differentiation. [61]

1.3 Collagen composition and metabolism in pelvic floor dysfunction

1.3.1 Changes in connective tissue in SI

In the late 80's Ulmsten reported that the skin of stress incontinence women contained 40% less collagen than that of the continent ones [62]. However the role of connective tissue alterations in incontinence is still not clear. Connective tissue is still being studied, probably with more sophisticated techniques, to try to establish the pathophysiology mechanism of this condition.

Several studies have supported Ulmsten's work. A lower hydroxyproline concentration and a higher concentration of estrogen receptors in the vesico-vaginal fascia from incontinent women was found when compared to continent controls [63]. Falconer et al cultured skin biopsies from stress incontinent and control women and compared the collagen production between both groups. There was a significant decrease in collagen

production in the stress incontinence group [64]. However when they studied paraurethral vaginal skin of premenopausal stress incontinent women they found a higher concentration of collagen whose fibres have a larger diameter. A higher level of mRNA of collagen I and III could indicate an alteration in collagen metabolism [65]. However they did not find any difference in the biochemistry or ultrastructure between incontinent and control women after the menopause [66]. In pubocervical fasciae of incontinent women collagen content is also diminished but no changes were found in collagenase activity, collagen solubility or cross linking [67].

In an interesting study which compared nulliparous young women with stress incontinence to controls, the study group was found to have less collagen, a decreased type I to III ratio and a significant reduction of the cross-linking content [68]. This study is significantly relevant because, having excluded childbirth as a cause for stress incontinence, it highlights the importance of connective tissue alterations in the pathogenesis of stress incontinence. Collagen III, which has elastic properties, was reduced in skin, uterosacral and round ligaments of women with stress incontinence +/- prolapse [69]. Collagen type I is reduced in pubocervical fascia of parous women with stress incontinence irrespective of their hormonal status [70]. Levels of mRNA for procollagen I and III in women with stress incontinence were lower than controls despite no demonstrable differences in collagen type [71]. Collagen morphology is also altered in women suffering stress incontinence [72].

1.3.2 Changes in connective tissue in prolapse

There are fewer studies that have analyzed collagen in pelvic organ prolapse. As in stress incontinence, collagen content in vaginal skin is found to be reduced in pelvic organ prolapse. Matrix metalloproteinases 2 and 9, enzymes responsible for collagen degradation, are increased in vaginal tissue affected by pelvic organ prolapse compared to controls. This would suggest an imbalanced collagen metabolism with a small proportion of newly synthesized collagen fibres but an overall increased degradation and therefore decreased strength [73]. Similar studies using molecular biology techniques in vaginal skin of women suffering from pelvic organ prolapse and stress urinary incontinence showed that messenger RNA expression of MMP1 was significantly increased compared to the controls and its inhibitors, TIMP 1 was reduced indicating a higher destruction of tissue in the incontinent group, suggesting increased collagen breakdown as a possible aetiology for stress incontinence and prolapse. Despite these studies it is difficult to know if these changes are actually the cause of the prolapse or the consequence [74]. Phillips et al studied collagen metabolism in uterosacral ligaments and vaginal tissue of women with and without uterovaginal prolapse and found a significant increase in pro MMP-2 in vaginal tissue in women with prolapse compared to controls. There was a significant correlation between pro-MMP2, activated MMP-2, MMP-9 and TIMP-2 in vaginal tissue and uterosacral ligament tissue which suggests that changes in vaginal tissue may be a result of prolapse rather than a cause [75].

A previous insult in the tissue could cause an alteration in the collagen metabolism, predisposing it to develop pelvic organ prolapse if further insults are received. At the

same time, an increase in the tension of the vaginal skin could be responsible for an increased collagen metabolism. Studying different areas of vaginal skin affected by prolapse could give us more information about the complex changes that happen in the tissue in women who develop pelvic organ prolapse.

1.4 Management of pelvic floor disorders

1.4.1 Treatment of stress incontinence

Conservative management

Conservative management should be the first line of treatment for stress urinary incontinence. Changes in life style, pelvic floor muscle training and bladder retraining are part of the treatment that should be offered. Symptoms of stress incontinence occur when there is a rise in the intraabdominal pressure. At the same time constant increased pressure in the pelvic cavity can be responsible for stress incontinence, so the life style changes are focused in the reduction of the insults which could be involved in the development and perpetuation of stress incontinence symptoms. These include weight loss, smoking cessation, caffeine reduction, balanced fluid intake, minimize heavy lifting or relief of constipation.

Several studies have shown that urinary incontinence is more prevalent in women with higher BMI (body mass index) than women with normal weight. Dwyer et al in a series of 368 incontinent women, found obesity was significantly more common in women with stress urinary incontinence than in the normal population [76] . The effect of weight loss has been studied mostly in morbidly obese women where their incontinence

symptoms improve after severe weight loss [77], [78]. A study analyzing the effect of weight loss in moderately obese women (BMI 38.3) demonstrated an association between weight loss and improvement in urinary incontinence symptoms [79]. Nevertheless the initial effects of weight loss have not been assessed long term if the weight loss is maintained.

The prevalence of urinary stress incontinence is slightly higher in young gymnastic women, athletes [80] and young soldiers [81] than in the normal population. This could just be the result of strenuous exercise which unmasks the symptoms. No randomized control trials have analyzed the effect of repetitive heavy lifting for a long period of time and the risk of SI but from clinical experience it seems there could be a relationship between them.

There is no clear evidence regarding the association between smoking and urinary incontinence but some studies suggest that smoking increases the risk of developing urinary incontinence. Smokers usually have increased risk of chronic cough and there is evidence that smoking has an effect on connective tissue. Both of these will have a direct impact on the pathophysiology of stress urinary incontinence.

Physical therapies

Physical therapies include pelvic floor muscle training, electrical stimulation, biofeedback, and the use of devices like vaginal cones.

Several randomized control trials have shown the superiority of pelvic muscle training versus no treatment for urinary stress incontinence with a success rate of 68 to 74%

[82;83]. Small studies have combined pelvic floor muscle training with other treatment methods like electrical stimulation or vaginal cones but the success rate seems not to differ significantly from the group treated only with pelvic floor muscle training alone. Electrical stimulation alone has not been proven to be superior to no treatment [84].

Several devices have been designed to avoid leakage of urine in women suffering from stress incontinence. Those include external collection devices, devices to support the bladder neck, occlusive devices, and intraurethral devices. There are no randomized controlled trials to assess their success rates. As the results from physiotherapy treatment are highly successful and surgery for stress incontinence is becoming less invasive these devices are probably aimed at women who want to avoid surgery or for those where previous therapies have been unsuccessful.

Medical treatment

A recent new serotonin (5-HT) and noradrenalin (NA) reuptake inhibitor, Duloxetine has been approved for the treatment of stress urinary incontinence. Duloxetine increases the concentration of 5-HT and NA in the sacral spinal cord in animal studies, increasing the activity of the external urethral sphincter and therefore preventing urinary leakage during the storage phase of the micturition cycle. Glutamine release determines the external sphincter muscle activity. During the voiding phase glutamine release is inhibited and there is no activity in the striated muscle of the external urethral sphincter, regardless of the presence of 5-HT or NA. Studies have shown an increase in Valsalva leak point pressure and in external urethral sphincter activity at rest and in coughing in women who responded to treatment. However the resting urethral pressure was not affected [85]. Duloxetine and pelvic floor muscle training are more effective than no

treatment and is more effective in reducing the number of incontinent episodes and improving the quality of life when compared to placebo [86].

Surgical treatment

When conservative treatment alone or in conjunction with medical treatment has failed to restore continence, surgical options should be the next line of treatment. There are several surgical techniques available with different degrees of morbidity and success rates. Selection of the patient is probably the most important step before deciding the surgical procedure to ensure the maximum success rate and minimize the morbidity. Urodynamic tests, symptom questionnaires, quality of life measurement, assessment of the patient's health and patient's expectations will determine which is the most appropriate treatment for that individual. The aim of the surgical treatment is to correct urethra hypermobility at the urethrovesical junction or strengthen the support of the tissues to restore internal sphincter function.

Intra-urethral therapy

This consists of the injection of a bulking substance in the urethral submucosa at the level of the bladder neck with the aim of narrowing the lumen of the urethra. The injections can be performed paraurethrally or transurethrally with the aid of a cystoscope. The latter technique allows a direct vision of the location of the needle and the bulk effect while the injection of the substance is taking place. It is a technique particularly designed for patients with intrinsic sphincter deficiency and stable detrusor muscle with a non mobile urethra. Intrinsic sphincter deficiency has been described in videourodynamics as the open bladder outlet in the absence of a detrusor contraction. Different substances have been used in the past with diverse success rates.

Polytetrafluoroethylene was the first agent to be commonly used. One of the problems with this substance was the migration of its particles and the granuloma formation. Glutaraldehyde cross-linked bovine collagen is a degradable substance which is slowly broken down by metalloproteinases. However, as it is substituted by living connective tissue some of the effectiveness is maintained in nearly 80% after 1year [87]. Success rates of intra urethral collagen injections are 64 to 95% [88]. Other substances used are autologous fat with poorer results than collagen. Sixty per cent is reabsorbed in only 3 weeks. Other substances are macroplastique and bioplastique which are silicone polymers. Materials currently in development are allogenic human collagen and autologous cartilage and synthetic agents include microballoon technology, hyaluronic acid with or without microsphere technology, hydroxylapatite and a variety of polymeric technologies [89]. Long term effects studies are lacking so it is difficult to evaluate the effectiveness of those treatments.

Needle suspension

The procedure consists in suspension of the paraurethral tissue to the rectus fascia with stainless steel wires. Various modifications of this technique developed in the following years but all of them follow the same principle. Results from these techniques give a success rate of around 70 to 75% at short term follow up [90], between 3 to 12 months. More disappointing are the long term results where at 5 years the success rate drops to 43% [91] and 33% at ten years [92].

Retropubic urethropexies

Burch in 1958 developed the, up to now, gold standard procedure for urinary incontinence with success rates much superior to any other technique available at that

time. It requires an abdominal (or laparoscopic) approach to reach the retropubic space. Three interrupted sutures are placed bilaterally in the lateral paravaginal fascia and attached to the Coopers ligament, the structure that acts as an anchor. The vagina wall is elevated without tension which will support the urethra lying above it. The short term results (12 to 24 months) are good ranging from 72 to 94% depending on the study and long term of 5 and 10 years vary from 63 to 89%.

Sling procedures

Again there are multiple variants of this surgical technique. Nevertheless all maintain the principle of introducing a strip of material suburethrally at the bladder neck which is attached to the abdominal wall or rectus fascia. Several different materials have been used as sling including organic and artificial autologous fascia lata, cadaveric fascia lata, homologous lyophilized dura mater, porcine dermis and porcine intestinal submucosa are some examples of organic materials used instead of rectus fascia as it facilitates the surgical procedure and reduces its morbidity. Synthetic materials have also been used. Mersilene, Vicryl, silastic, gore-tex, Teflon, Prolene and Marlex are examples of them. These materials have higher tensile strength than the organic material but have a higher rate of mesh erosion.

A new sling technique, the tension free transvaginal tape (TVT), was developed in 1994 by Ulmsten [93]. One of the principle differences between the old sling procedures is that TVT is designed for the mid-urethra instead of the bladder neck. Two small (1cm) abdominal incisions are made just above the pubic bone but the approach is mainly through a small vaginal incision at the level of the mid urethra. The paraurethral spaces are dissected bilaterally. The Prolene tape is attached to two curved needles at

both ends. One needle is passed through the one side of the dissected paraurethral space and the second one through the other side and then through the retropubic space appearing just above the pubic bone. The sling is left tension free and it is not sutured to any structure as it fixes to the tissues thanks to its mesh characteristics. A randomized control trial comparing colposuspension versus TVT showed similar success rate for both procedures but the hospital stay and the time to go back to work was significantly reduced in the TVT group. Long term outcome at 5 years is 81% in a Scandinavian study [94] and 94% in a recent Korean study [95].

Newer variations of the TVT are developing. Transobturator tape (TOT) is one of them. Instead of using the retropubic space in this technique the tape is inserted from the inguinal region through the Obturator fossa into the paraurethral space. A recent randomized study comparing TOT with TVT showed similar results at 12 months for both techniques [96]. TOT could have the advantage of less hemorrhagic complications, although more research is needed.

1.4.2 Treatment of pelvic organ prolapse

Conservative treatment

Vaginal pessaries are the most common non-surgical treatment for pelvic organ prolapse. They are the treatment of choice when surgical options need to be avoided. When placed in the vagina they support the bladder, rectum or the uterus. They have been demonstrated to improve not only the symptoms of prolapse in the vagina but also the urinary and bowel symptoms that can go with cystoceles and rectoceles [97].

There are several types of pessaries, mainly made of latex, rubber silicone or acrylic. Examples of those are:

- Lever: Hodge, Smith-Hodge and Risser.
- Ring
- Shaatz
- Gellhorn
- Inflato-ball
- Cube
- Donut

Complications of pessaries include:

- Vaginal discharge
- Vaginal bleeding
- Vaginal mucosa erosion and pessary incarceration
- Urinary retention
- Urinary tract infection

Surgical treatment

Anterior compartment

White (1911) described that the cure of a cystocele should include reapproximation of the lateral vaginal fornices to the arcus tendineus. Kelly described the plication technique and midline plication for cystocele repair. In 1976 Richardson published his classification of fascial defects. He described lateral, transverse (superior), and midline defects in the pubocervical fascia. This classification explains the need for different types of repair depending on the site of the specific defect. Paravaginal defects have been defined as detachment of the endopelvic fascia from the arcus tendineous fascia

pelvis and were found in 67% of patients with anterior vaginal prolapse. Paravaginal repair techniques were developed to repair this type of prolapse and those can be performed via the vaginal or the abdominal route. Shull et al [98] defend the former technique while Benson et al [99] support the abdominal route after having found a delay in the pudendal and perianal nerve terminal motor latency after vaginal dissection. Different surgical techniques use mesh to reinforce either anterior or posterior vaginal wall to reduce the risk of recurrence. Nevertheless they are associated with an increase in surgical complications, like mesh erosion, fistula and dyspareunia.

Anterior colporrhaphy

The aim of the anterior colporrhaphy is to plicate the muscularis and adventitia of the vaginal skin in the midline to create strong support to reduce the protusion of the bladder and vagina with interrupted stitches. The vaginal epithelium is then excised and sutured with a continuous suture with delayed absorbable material. One modification is to extend the dissection laterally up to the inferior pubic rami. Stitches are placed laterally in the paravaginal tissue and plicated under tension. This technique can narrow the vagina to a greater extent. Another modification involves the use of a synthetic or organic mesh after the plication of the vaginal muscularis to create an extra support.

Raz et al [100] developed a new combined technique to repair large anterior vaginal wall prolapse (grade IV cystourethrocele). This includes an anterior colporrhaphy to correct the central defect of the cystocele and repair of the paravaginal herniation of the bladder and bladder neck by a needle suspension of these structures.

Vaginal paravaginal repair

The aim of a paravaginal repair for correction of cystocele is to attach the detached lateral vagina at the level of the arcus tendineus. The anterior wall of the vagina is incised in the midline and dissected laterally up to the space anteriorly along the inferior pubic ramus medially to the pubis and laterally to the ischial spine. Four to six stitches will be placed in the white line, into the pubocervical fascia and into the undersurface of the vaginal epithelium. Subsequent stitches move posteriorly towards the ischial spine where the later stitch is attached to the vaginal apex. The vaginal muscularis can be plicated in the midline. Redundant epithelium is excised before closure with absorbable sutures.

Abdominal and laparoscopic paravaginal repair

There are few studies in the literature studying abdominal paravaginal repair. Scotti et al [101] introduced the concept of a "safe zone" to place the most cephalad suture; an area on the medial surface of the ischial spine extending anteriorly and caudally to the inner surface of the pubic bone and obturator membrane. They recruited 40 women with lateral vaginal wall detachment. Paravaginal fixation to the ischial periosteum and obturator membrane was performed. He passed the suture through the arcus tendineus and obturator internus muscle, piercing the periosteum and obturator membrane after careful palpation of the ischial spine and sciatic foramen to ensure safe and anatomically correct suture placement. He reported recurrence rates of 2.5 % for the lateral vaginal wall and 8.5% for the anterior vaginal wall at an average of 39 months follow up.

Laparoscopic paravaginal repair seems to be a safe technique with a similar success rate [102]. Complications rates have been reported between 2 to 4%. Average blood loss has been reported as 50 ml, average length in hospital of 23 hours and average surgical time of 70 min [103].

Posterior compartment

Several surgical techniques have been reported in the literature. There are two very different techniques using different routes to repair posterior vaginal wall defects, the vaginal route which is preferred by gynaecologists and the transanal route performed mainly by colorectal surgeons.

Posterior colporrhaphy

Posterior colporrhaphy is performed through a midline diamond shaped incision in the posterior vaginal wall. A row of interrupted sutures are placed in the rectovaginal fascia and a second layer of interrupted stitches plicates the margins of the levator ani muscles. The posterior vagina mucosa is trimmed and closed with absorbable sutures. This technique has been associated with high incidence of dyspareunia postoperatively.

Transverse repair

Marek et al [104] developed a modified transanal technique to repair rectocele. By inserting one finger in the rectum the margins of the rectocele could be delineated. Allis clamps were placed laterally and traction was applied and the mucosa was removed from one side to the other, superiorly and inferiorly until the mucosa over the rectocele was exposed. The wound is closed with a continuous lockstitch suture that penetrates

through the mucosa and the perirectal fascia and may include the superficial layer of the muscular of the rectum.

Transrectal repair

An incision of the anterior rectal mucosa is made 1 cm above the dentate line. Dissection is performed anteriorly and laterally. The area including the rectal muscle and the rectovaginal septum is plicated using interrupted sutures. A second layer is used to close the mucosal defect.

Laparoscopic rectocele repair

Lyons et al[105] in a prospective study evaluated 20 women who underwent laparoscopic rectocele repair using a polyglactin mesh. He followed them up at 3 monthly intervals for a year. The mean operating time was 35 minutes and the hospital stay was less than 24 hours. Eighty percent of the patents had symptomatic relief of digitation and prolapse at 1 year.

Fascial repair

Dennvilliers, Uhlenhuth, Milley and Nichols support and demonstrated the existence of a rectovaginal septum and found that in cystoceles there were breaks in this tissue layer they called pubocervical fascia. Richardson explained the existence of a defect in the rectovaginal fascia causes rectoceles. Richardson was among the first gynaecologists to advocate reapproximating these fascial breaks to restore original anatomy integrity. The fascial repair is described with a diamond shaped incision at the introitus. Reflection of the vaginal mucosa is performed extensively laterally. With one finger in the rectum the edges of the connective tissue should be grasped and pulled

over the area of bare rectal wall. The edges are plicated with interrupted sutures and redundant skin is excised and sutured. If the septum is detached from the perineal body it is reattached.

All surgical techniques described have an important limiting factor and it is the use of the women's own tissues to restore anatomy. The presence of pelvic organ prolapse indicates already the existence of some degree of tissue damage. The degree of the prolapse and the quality of the tissue play an important role in surgical success rate and recurrence. This could explain the high recurrence rate after surgical repair. When tissues are very poor a mesh can be placed to increase the support. Different types of meshes have been used in the anterior and the posterior vaginal wall to add extra support at the time of surgery including synthetic and organic ones. Both have increased risk of infection, erosion and dyspareunia despite been higher in the synthetic group. Therefore this tends to be an option for recurrent cases or elderly patients who are not sexually active.

As we do not have an optimal management option for pelvic organ prolapse, prevention becomes even more important. Improving the quality of the tissues or detecting women susceptible to developing pelvic organ prolapse would help in reducing the incidence of pelvic floor dysfunction and therefore improve the quality of life of a large number of women. To achieve that it is necessary to improve our knowledge and understanding of the pathophysiology of pelvic floor dysfunction.

1.5 Hypothesis and objectives

This research project is a study of pelvic floor pathology from a molecular point of view to improve our understanding of its pathophysiology. As seen previously in the introduction, chapter 1, changes in connective tissue have been detected in vaginal skin and pelvic fascia of women suffering from pelvic organ prolapse and stress incontinence. Several theories regarding pathophysiology mechanisms and etiological factors have been established but we still do not have a clear understanding of why stress incontinence and pelvic organ prolapse happen to some women and not to others.

The present project was divided into two lines of research, one studying the connective tissue of women with pelvic organ prolapse (Stretched and non-stretched vaginal tissue in white women with prolapse) and a second dedicated to the analysis of connective tissue in women with stress incontinence from different ethnic background (Stress incontinence in different races study). Differences in connective tissue could give a clue to the discrepancies observed in the prevalence of stress incontinence and prolapse between white and black races.

Increased MMPs have been found in vaginal skin of women with pelvic organ prolapse in previous studies (refs). Nevertheless these studies are not able to establish if these findings are the cause or the consequence of pelvic organ prolapse. Our prolapse study was designed to try and answer this question. Vaginal skin affected by pelvic organ prolapse was studied to determine the affect of strain (stretched and non-stretched vaginal tissue) on the collagen metabolism and give us a better

understanding of the pathophysiology mechanism contributing to the development of pelvic organ prolapse.

Reduced prevalence of stress incontinence has been observed in black women compared to white Caucasian. We studied connective tissue characteristics in black and white women to identify any difference which could explain this discrepancy. This could help us understand the mechanism leading to the development of stress incontinence and identify protective factors in black women which could be useful for the prevention and treatment of stress incontinence.

1.5.1 Hypothesis

a) Stress incontinence in different racial study

Differences in collagen metabolism or composition will be found in black women compared to white women. Those differences could explain the lower incidence of stress incontinence in the black population.

b) Stretched and non-stretched vaginal tissue in white women with prolapse

Stretched vaginal prolapse tissue will show differences in collagen degradation (MMP2) from non-stretched vaginal prolapse tissue which could explain the effect of tissue stretching in collagen metabolism. Differences in adhesiveness of the tissue (collagen XVII) will be shown between stretched and non stretched tissue.

All prolapse tissue will show an elevation in collagen degradation compared with stress incontinence alone.

1.5.2 Objectives

1. To study vaginal tissue from controls and women with stress incontinence, from Caucasian and Black African origin.

Aims include:

a To measure collagen I and III by immunohistochemistry

b. To assess collagen metabolism by measuring enzymes implicated in the degradation of collagen (total and active MMP 2) using enzyme immunoassay techniques.

c To measure Collagen XVII expression, as an indication of adhesiveness of the tissue, using molecular biology

2 To study stretched and non-stretched vaginal tissue in Caucasian women with prolapse.

a To measure total MMP-2, active MMP-2 and TIMP-2 by Enzyme immunoassay techniques as indicators of collagen metabolism or remodelling.

b To measure collagen XVII using molecular biology as an indication of alterations in adhesiveness of the tissue.

METHODOLOGY

Chapter 2:

Tissue sampling

Tissue sampling

Stress incontinence in different races study

This study required the collection of vaginal skin from women with and without stress urinary incontinence from two different races, black and white, to study tissue properties that could explain the reduced prevalence of stress urinary incontinence in black women. The design of the study involved two centres, one in South Africa where the collection of black and white samples took place and the second one in the United Kingdom, where the analyses of the samples were performed. As the prevalence of stress incontinence in black women is very low, South Africa guaranteed a high density of black population to ensure the completion of recruitment in a reasonable period of time.

The study protocol, data collection questionnaire and transport of the samples was organised in England. The samples were collected and stored at -80°C or fixed and embedded in paraffin until arranged for transport in dry ice or room temperature as appropriate. Samples were coded so that the analyses were blinded.

2.1 Ethical Approval

Ethical approval was obtained by the Tygerberg Hospital Ethical Committee in Cape Town, South Africa (project number 2003/033). Ethical approval was also requested

and obtained by Southampton University Hospital Trust Ethical Committee (REC reference 367/02/w).

2.2 Selection of patients

Patients were recruited from the Gynaecologic Clinic in Cape Town. Women with benign gynaecological conditions, different to urinary incontinence, and women suffering from stress incontinence needing a surgical procedure were asked to participate in the study. Women who accepted and fulfilled the inclusion and exclusion criteria completed a data collection questionnaire designed exclusively for the study see Appendix 1.

2.3 Data collection

Data including weight, diet, smoking habit, social and educational status were collected. Past obstetric history (parity, mode of delivery, birth weight, performance of pelvic floor exercises), gynaecological history (hormonal status, previous gynaecological surgery and urinary and pelvic organ prolapse symptoms) and relevant medical history were recorded (chronic cough, chronic constipation, connective tissue disorders, diabetes, haemorrhoids, varicose veins, urinary tract infections, CNS disorders, spinal cord injuries, congenital disorders, genitourinary anomalies, abdominal hernia, PID, degenerative fibroids, malignancy, stroke and ligament injury). Previous pelvic surgery for incontinence and prolapse, hysterectomy and pelvic irradiation were recorded.

Regular medication was recorded and special attention was paid to diuretics, alpha blockers and ACE inhibitors for their affects on the urinary system. Allergies were also noted.

Urinary symptoms were recorded in the following table:

Symptoms	**Y / N**	**Duration**	**Severity** (n° of times a day)	**Comments**
Stress incontinence	Y / N			
Urgency	Y / N			
Urge incontinence	Y / N			
Frequency	Y / N			
Nocturia	Y / N			
UTI	Y / N			
Prolapse symptoms	Y / N			

Table 2.1 Table included in the medical and examination questionnaire used to record the symptoms, duration and severity of the urinary tract from the data collection questionnaire.

A complete abdominal examination was performed to exclude any palpable abdominal masses. Vaginal examination was performed to assess the quality of the vaginal skin (normal, red, sore, rash and excoriated) and the vaginal mucosa (well estrogenised, poorly estrogenised and atrophic). Perineum was inspected and classified between healthy, scarred and deficient. A bimanual vaginal examination was performed to detect any pelvic organ prolapse and demonstrate stress incontinence.

Pelvic organ prolapse was assessed following the ICS classification:

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

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

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

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

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

Any joint hypermobility and abdominal stria were noted. All women had a blood test to exclude HIV or hep B infection.

Before surgery, women with stress incontinence symptoms had Urodynamic studies done as part of their preoperative assessment. Urodynamics were performed in a standardised way following the ICS guidelines. Urodynamics studies included initial uroflowmetry, which measures the flow rate and cystometry, which measures the pressure/volume relationship in the bladder. Uroflow studies were performed to exclude any obstruction of the outflow tract. Cystometry measures the detrusor pressure during controlled bladder filling and subsequent voiding with measurement of the synchronous flow rate. Cystometry assesses detrusor activity, sensation capacity and compliance.

Urodynamics studies were commenced with uroflow recording. The bladder was then catheterised with a filling catheter plus a pressure catheter to check for any residual urine and provide readings of bladder pressure throughout the cystometry; to provide an abdominal pressure for subtraction a pressure balloon in the rectum was used. External transducers at the level of the synphisis pubis were zeroed to atmospheric pressure. Bladder was filled at a rate of 50ml/minute. Maximum volume inserted was 400 ml or lower according to the maximum volume tolerated by the woman. The first sensation of bladder filling, first desire to void and strong desire to void and the maximum cystometric capacity were recorded. Detrusor function was assessed during the filling phase. Cough test was performed to objectivise any urinary leakage. Urodynamic stress incontinence is defined as the involuntary leakage of urine during increased abdominal pressure, in the absence of a detrusor contraction. Urine flow was studied at the final stage of the urodynamic studies and included maximum flow rate, average flow rate, voiding times, bladder capacity, maximum bladder pressure, residual and leak point.

Women who had no demonstrable urodynamic stress incontinence were not included in the study.

2.4 Inclusion and exclusion criteria

Inclusion criteria were:

- Age between 18 and 85 years.
- White African or Black African as ethnic group.

- Women suffering from a benign gynaecological condition (different to urinary incontinence) and women with stress incontinence requiring surgery.
- Demonstrable genuine urinary stress incontinence with stable bladder in Urodynamic test.
- Women able to give written consent.

Women with benign gynaecological conditions were recruited to act as controls and women with stress incontinence formed the study group.

Exclusion criteria were:

- Women younger than 18 and older than 85 years old.
- Other ethnicity apart from white and black.
- Women with symptoms of stress urinary incontinence but not proven at urodynamic test or women with urodynamic diagnosis of overactive bladder.
- Women with any of the following past medical history:
  - chronic cough
  - chronic constipation
  - connective tissue disorders
  - diabetes
  - haemorrhoids
  - varicose veins
  - urinary tract infections
  - CNS disorders
  - spinal cord injuries

  - congenital disorders
  - genitourinary anomalies
  - abdominal hernia
  - PID
  - degenerative fibroids
  - malignancy
  - stroke
  - ligament injury
- Women with previous surgical history of prolapse or incontinence surgery.
- Women with previous history of irradiation.
- Women taking any of the following medication:
  - Diuretics
  - ACE inhibitors
  - Alpha blockers
- Women HIV or hepatitis B positive.
- Women not able to sign the consent form.

2.5 Sample collection and transport

Samples were obtained in the operating theatre during the surgical procedure. A small biopsy measuring 10mm x 10mm x 10mm approximately from paraurethral vaginal skin was obtained from each woman.

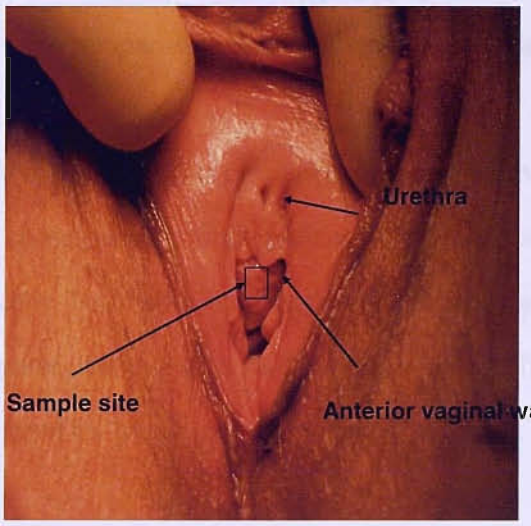


Figure 2.1 Diagram illustrating the site from which the paraurethral vaginal skin sample was taken for the stress incontinence in different races study

Where possible, samples were divided in three pieces and two of them were immediately frozen in liquid nitrogen. Samples were then transported to the laboratory and stored in the -80 °C freezer until transport. The third sample was embedded in paraffin blocks and stored at room temperature.

Three transportations were organised in total from South Africa to the UK. The transport of the samples was organized from the UK by a courier service. Samples were collected from the laboratory of Tygerberg Hospital in Cape Town and stored in dry ice for the transport and sent to England by air. Once the samples were cleared through Customs, in the UK, they were checked to guarantee the presence of dry ice and to confirm that the samples were still frozen. Some further dry ice was added to them if necessary. Then the samples where transferred to Southampton by road.

Samples were stored at -80°C until analyses was performed. The paraffin blocks were transported and kept at room temperature until required for analysis.

Samples were coded with a number. This number was recorded on the data collection sheath or questionnaire corresponding to the patient. The questionnaires were sent with the samples, in a folder. This folder was kept by the supervisor of the project and not shown to the investigator until analyses had been carried out. This way the investigator was always blind for group (control or study) and race.

Stretched and non-stretched vaginal tissue in white women with prolapse

The study aimed to obtain tissue samples from vaginal skin of women suffering with pelvic organ prolapse (cystocele or rectocele) from two different areas and analyse the differences in markers of collagen metabolism which could help us understand the physiopathology of pelvic organ prolapse.

2.6 Ethical approval

Ethical approval was obtained by Southampton and South West Local Research and Ethics Committees, submission number 074/98.

2.7 Selection of the patients

Patients were selected from the waiting list for pelvic organ prolapse (anterior or posterior colporrhaphy) from Princess Anne Hospital. Patients who fulfilled the criteria were invited to participate in the study and a consent form was signed. Data regarding

age, occupation, weight, smoking habit, parity, mode of delivery, hormonal status, hormone replacement therapy and topical estrogen use was recorded.

2.8 Inclusion and exclusion criteria

Inclusion criteria were:

- All patients with a second or greater degree cystocele or rectocele according to ICS classification.
- Age above 18 years

Exclusion criteria were:

- Those patients who at the time of the surgery were found not to have tissue that conserved the normal vaginal folds
- Previous history of cancer
- First degree pelvic organ prolapse

2.9 Sample collection

Two samples were obtained from each patient at the time of surgery. One sample was obtained from the area of vaginal skin most affected by the stretching, usually at the point of most protusion into the vagina of the prolapse. This sample was labelled as "stretched". A second sample was obtained from an area of vaginal skin affected by the prolapse but still maintained the normal folds of the vaginal skin. This area was not affected by the maximum stretching and therefore was labelled as "non-stretched". As

both samples were obtained from the same women, the "non-stretched" sample acted as a control for the "stretched" group.

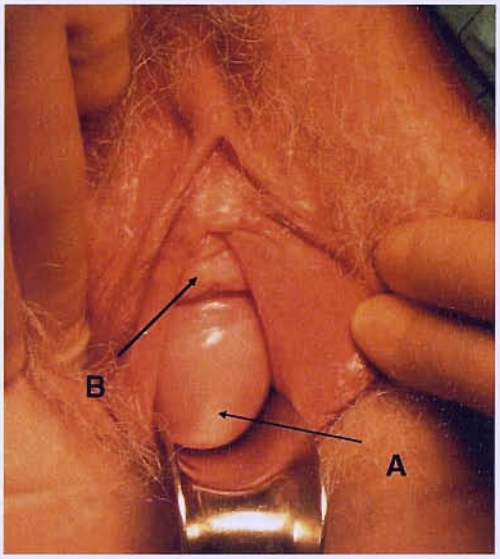


Figure 2.2 Representation of the area where samples were obtained in the stretched and non-stretched vaginal tissue in white women with a cystocele included in the prolapse study. (A) Represents the stretched sample and (B) represents the non-stretched sample.

Samples measured approximately 10x10x10 mm and were immediately frozen in liquid nitrogen after collection. Samples were labelled using the initials of the patient and "S" for the stretched group and "NS" for the non-stretched samples. Samples were stored in a container of liquid nitrogen until the analyses were performed. Fifteen women were recruited and eleven sets of samples were obtained but only eight sets of samples were suitable for the analyses. Two sets of samples had to be excluded as at least one of the paired samples did not achieve the weight required for analyses. A third sample had to be excluded as minimal levels of RNA were found during quantification after the process of total RNA extraction was completed.

METHODOLOGY

Chapter 3:

Total protein extraction

Total protein extraction

The same protein extraction technique was used for samples of the prolapse study and the racial study so the description of the method is the same for both studies.

3.1 Techniques for homogenizing samples for protein extraction

3.1.1 Cutting of the samples

Samples were removed from the -80°C freezer and stored in dry ice in a box to avoid thawing. Cutting of the samples was performed on a Petri dish lying on a layer of dry ice to keep the temperature down to -80°C. A small tissue holder and the cutting blade were also cooled down by leaving them on dry ice for few minutes before use.

Samples were removed from their containers and cut in small pieces. Individual pieces were weighed using a highly sensitive balance. Samples weighed between 28 and 32 mg.

Weight was recorded and the samples were stored individually in different containers appropriately labelled and which had previously been cooled down by storing them on dry ice for a few minutes.

3.1.2 Homogenisation techniques

Samples were homogenized using Dulbecco's Phosphate Buffered Saline (PBS) at ph 7.4. The solution contained:

- $Ca\ Cl\ 2H_2O$ 0.132 g/l
- $K\ Cl$ 0.20 g/l
- KH_2PO_4 0.20 g/l
- $Mg\ Cl_2\ 6H_2O$ 0.10 g/l
- $Na\ Cl$ 8.00 g/l
- $Na_2\ HPO_4$ 1.15 g/l

Triton X100 was added to the Phosphate Buffered Saline solution. Triton is a detergent which increases protein solubility and therefore facilitates the extraction of relevant proteins from the tissue into solution during homogenisation. However, high concentrations of Triton can interfere in the protein assay. Therefore, the optimal concentration of triton for homogenisation of the samples without interfering with the results of the Protein Assay (Coomassie Plus) was calculated using varying dilutions of triton in PBS. Negative controls (no sample added) were used. The concentrations used were: 0.5%, 0.25%, 0.125%, 0.06%, 0.03%, 0.015% and 0.007%. The three highest concentrations of triton interfered with the assay while the 4 lower concentrations did not, therefore, a final concentration of 0.05% was used, which solubilised appreciable amounts of protein of interest over and above buffer without Triton.

Triton at this concentration was later reported by the manufacturers to be compatible with the protein assay protocol used for protein analyses (Coomassie Plus).

The volume of the PBS solution added to the sample for homogenisation was determined from the weight of the samples and 0.5ml were used per 30mg of tissue.

Total volume of PBS/Triton = 0.5 ml of PBS/Triton x weight of tissue (mg)/30

Homogenisation was performed using a Labour technik T8.01 Ultra-Turrax homogenizer by IKA-WERKE, GMBH & Co.KG, (Staufen, Germany). The samples were homogenised for 30 seconds and then cooled on ice for 1 min. The procedure was repeated until homogenisation of the whole tissue was achieved and a homogeneous sample obtained.

The solution was centrifuged at 10000 rpm for 5 min. The supernatant was removed for assay (400µl approximately).

3.2 Protein extraction

Coomassie Plus™ Assay (The Better Bradford Assay Reagent) was used for measurement of total protein concentration. It is a Coomassie-binding colorimetric method. When Coomassie dye binds protein in an acidic medium, there is an immediate shift in absorption maximum from 465 nm to 595 nm with a concomitant colour change from brown to blue.

3.2.1 Preparation of the standards

Bovine Serum Albumin (BSA) is used to create the standard curve of known protein concentrations and from which the unknown samples protein concentrations are calculated.

One ml arnpoules of BSA (2mg/ml) were used to prepare the standards.
The standards were prepared by doubling dilutions. Five hundred µl of PBS with 0.05% triton were added to six tubes labelled as S6, S5, S4, S3, S2 and S1. Tube labelled as S7 contained 1 ml of 2mg/ml BSA. Five hundred µl of BSA from tube 7 were removed and transferred to tube S6. The tube was vortex mixed and centrifuged at 1000 rpm for five seconds. Five hundred µl of the mixture were removed and transferred to tube S5. This was mixed and centrifuged. The same procedure was repeated successively until obtaining 1 ml of 0.03125 mg/ml in tube S1. Tube S0 contained only PBS with triton without BSA. The concentrations were the following:

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
S7	0 µl	1000 µl of Stock	2 mg/ml
S6	500 µl	500 µl of vial S7dilution	1 mg/ml
S5	500 µl	500 µl of vial S6 dilution	0.5 mg/ml
S4	500 µl	500 µl of vial S5 dilution	0.25 mg/ml
S3	500 µl	500 µl of vial S4 dilution	0.125 mg/ml
S2	500 µl	500 µl of vial S3 dilution	0.0625 mg/ml
S1	500 µl	500 µl of vial S2 dilution	0.03125 mg/ml
S0	500 µl	0	0 mg/ml

Table 3.1 Example of dilution of the standards to create the standard curve

This dilution proved to be reproducible and sensitive for requirements of the assay. The standard curves were calculated initially manually. A computer program was run to obtain the standard curve from the plate reader. The standard curves were found to not be significantly different and therefore the computer program was used for further analyses and calculation of unknown results.

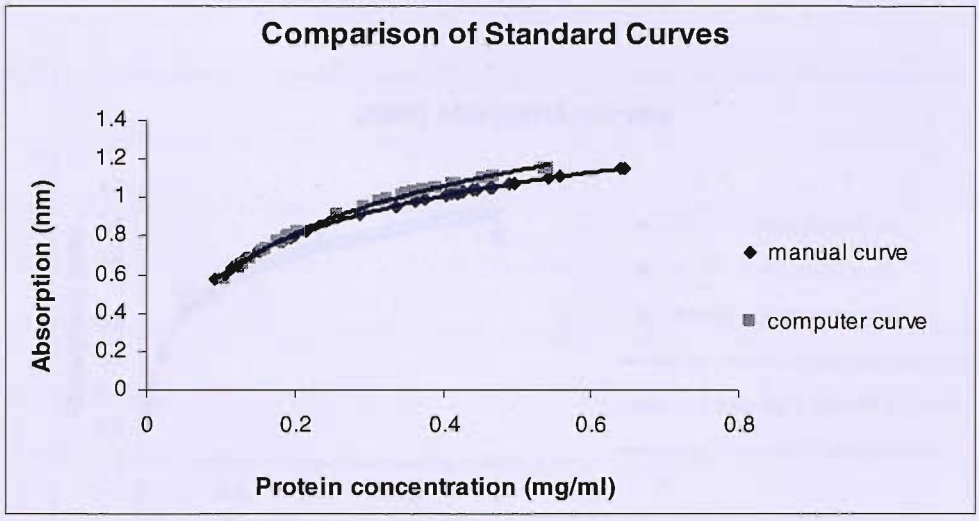


Figure 3.1 Comparison of standard curve obtained manually and using the computer program.

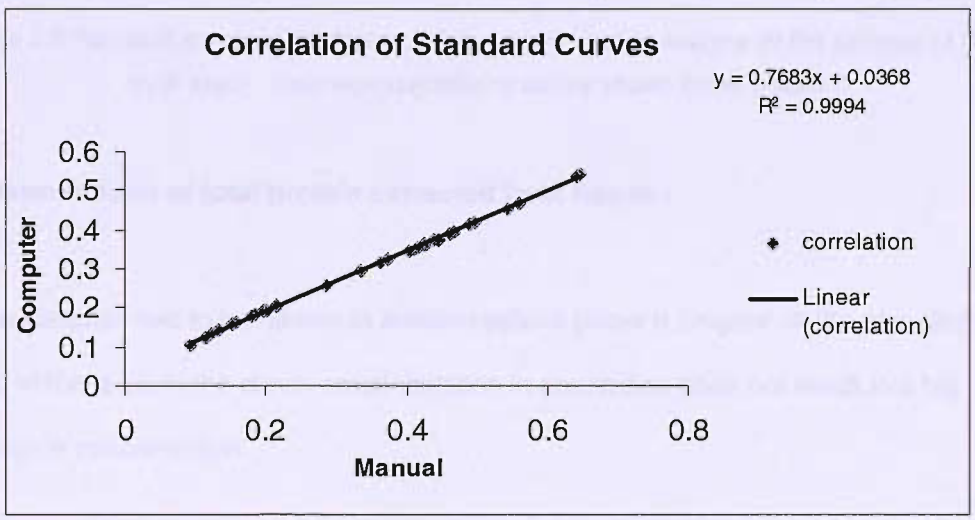


Figure 3.2 Correlation of standard curves obtained manually and using computer program.

As the assays correlated well and computer calculation was more convenient, the computer program was used for further assays.

The three standard curves used for the protein assays are shown in the next graph (figure 3.3).

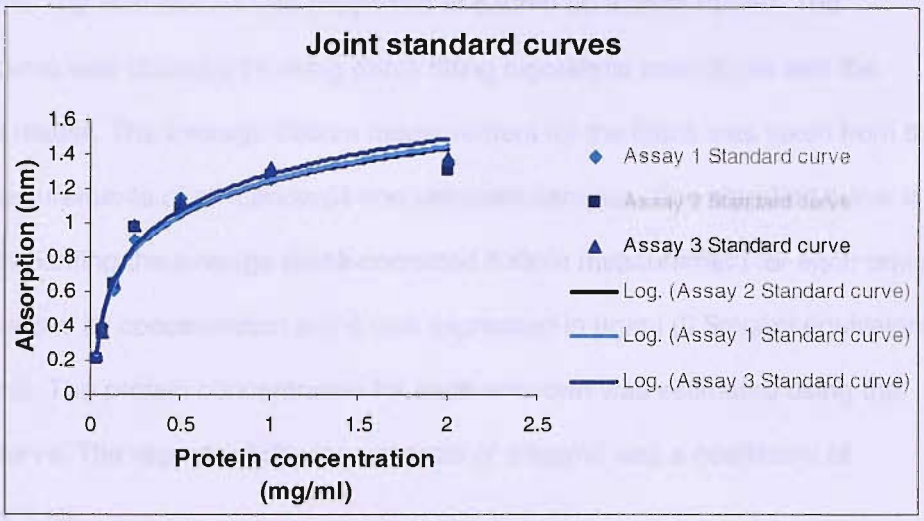


Figure 3.3 Standard curves of the three protein assays used to analyse all the samples of the racial study. Their reproducibility is clearly shown in the graph.

3.3 Measurement of total protein extracted from tissue

Tissue samples had to be diluted to achieve values below 0.5mg/ml on the standard curve. In this area of the curve, small variation in absorption does not result in a big variation in concentration.

Three different random samples diluted at 1/2, 1/4, 1/8 and 1/16 were tested to determine the most optimal dilution of samples for the assay. The optimal dilution was 1/8.

Microtitre plates were used to analyze the samples. Fifty µl of the standard and the unknown sample were pipetted in to the appropriate wells of the plate in duplicates. Two hundred and eighty µl of Coomassie Plus Reagent were added to each well and

mixed with a plate shaker for 30 sec. The plate was incubated for 10 min at room temperature. The absorbance was measured at 630nm on a plate reader. The standard curve was obtained by using curve fitting algorithms associated with the microplate reader. The average 630nm measurement for the blank was taken from the 630nm measurements of all standards and unknown samples. The standard curve is obtained by plotting the average Blank-corrected 630nm measurement for each protein standard versus its concentration and it was expressed in µg/ml (0.5mg/ml equivalent to 500µg/ml). The protein concentration for each unknown was estimated using the standard curve. The reproducibility for a sample of 93µg/ml was a coefficient of variation of 3.5%.

METHODOLOGY

Chapter 4

Enzyme immunoassay techniques

Enzyme immunoassay techniques

4.1 Introduction

As described in the introduction (chapter 1), collagen is degraded by a family of enzymes called matrix metalloproteinases (MMP). Degradation and remodelling of the tissues is regulated by a balance between these and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPS). Additionally, regulation can come from MMP message and protein production at the cellular level to make them more available and then at the extracellular level by activating their enzyme action.

MMPs are released as a pro active form. This enzyme is activated in the extracellular matrix. TIMPs can inhibit the active and the pro active form of MMPs. MMPs can be measured in the tissues in their active form, pro-MMP form and the MMP complex inactivated by the TIMP.

4.2 Preparation of the samples

4.2.1 Cutting of the samples

Samples were removed from the -80ºC and stored in a box in dry ice to avoid thawing. Cutting of the samples was performed on a cooled Petri dish lying on a layer of dry ice.

The small tissue holder and the blade were cooled down on dry ice for few minutes before use.

Samples were removed from their containers and cut in small pieces. Individual pieces were weighed using a sensitive balance. Samples weighing between 28 and 32 mg were used.

Weight was recorded and the samples were stored individually in cooled and appropriately labelled containers.

4.2.2 Homogenisation techniques

Samples were homogenized using Dulbecco's Phosphate Buffered Saline (PBS). The solution contains:

- $Ca\ Cl\ 2H_2O$ 0.132 g/l
- $K\ Cl$ 0.20 g/l
- KH_2PO_4 0.20 g/l
- $Mg\ Cl_2\ 6H_2O$ 0.10 g/l
- $Na\ Cl$ 8.00 g/l
- $Na_2\ HPO_4$ 1.15 g/l

Triton was added to the Phosphate Buffered Saline solution in a concentration of 0.05%.

The volume of the PBS solution added to the sample for homogenisation was determined by the weight of the samples (0.5ml buffer/30mg tissue).

Homogenisation was performed using a Labour Technik T8.01 Ultra-Turrax homogenizer by IKA-WERKE, GMBH & Co.KG, (Staufen, Germany) at 13,000 rpm. The samples were homogenised for 30 seconds and then left for few minutes in dry ice. The procedure was repeated until complete homogenisation.

The solution was centrifuged at 10000 rpm for 5 min and the supernatant was removed (400 µl approximately). This supernatant was kept at -20 °C until used for the different ELISA assays. This material was also used for the protein assay described in Chapter 3.

4.3 Total matrix metalloproteinase-2 ELISA Assay

The Biotrak MMP2, human, ELISA system from Amersham Biosciences provides a specific and precise quantitative determination of human MMP2. The assay is based on a two site ELISA sandwich format. Standard and samples are incubated in microtitre wells precoated with anti-MMP2 antibody as shown in the figure below.

The term "total" MMP2 has been used to describe the results of this assay although it is recognised that the data does not include the small proportion which is "active" enzyme.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S0	3	3	11	11	19	19	27	27	35	35
B	S1	S1	4	4	12	12	20	20	28	28	36	36
C	S2	S2	5	5	13	13	21	21	29	29	37	37
D	S3	S3	6	6	14	14	22	22	30	30	38	38
E	S4	S4	7	7	15	15	23	23	31	31	39	39
F	S5	S5	8	8	16	16	24	24	32	32	40	40
G	1	1	9	9	17	17	25	25	33	33	41	41
H	2	2	10	10	18	18	26	26	34	34	42	42

Figure 4.1 Example of standards and samples dispensed in the wells microplate (S0 to S5 represent the standards. Samples are numbered from 1 to 42 in this example). Samples are dispensed in duplicate.

Any MMP2 present is bound to the wells other components of the samples are removed by washing and aspiration. A second antibody with peroxidise bound to it is incubated in a second incubation (see figure below). After further washing, the amount of peroxidase bound to each well is determined by the addition of TMB substrate. The reaction is stopped by addition of the acid solution, and the resultant colour read at 450nm in the microtitre plate spectrophotometer. The concentration of MMP2 in a sample is determined by interpolation from the standard curve.

The assay recognises pro-MMP2 (free form and complexes with TIMP2). It does not recognise active MMP2.

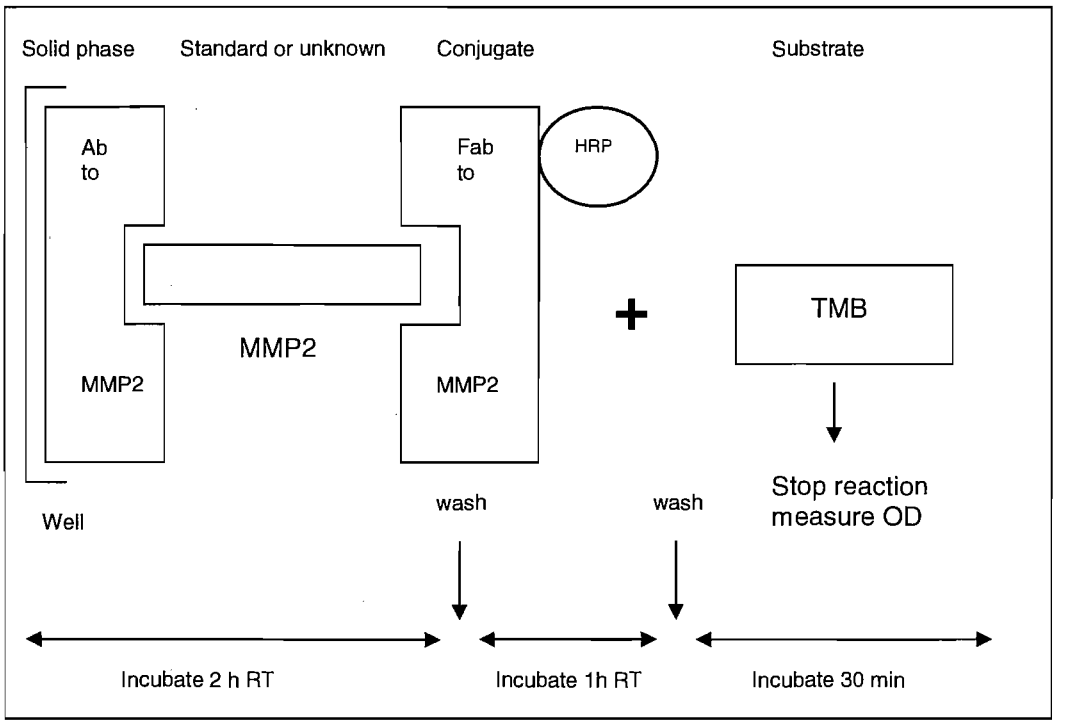


Figure 4.2 MMP-2 ELISA assay design

4.3.1 Preparation of the reagents

Assay buffer

The assay buffer supplied was transferred to a 100 ml measuring cylinder by repeated washing with distilled water. The final volume was adjusted to 100 ml with distilled water and mixed thoroughly.

Standard

One ml of distilled water was added to the bottle containing the standard and it was gently mixed until the content was dissolved.

Peroxidase conjugate

Twelve ml of distilled water was added to the bottle and mixed until the content was completely dissolved.

Wash buffer

The wash buffer was transferred to a 500 ml cylinder by repeated washings with distilled water. The final volume was adjusted to 500 ml with distilled water and mixed thoroughly.

4.3.2 Preparation of standards

Five polypropylene tubes were labelled as 1.5, 3, 6, 12 and 24 ng/ml. Five hundred µl of the assay buffer were dispensed in each tube. Five hundred µl of the stock standard (48ng/ml) were pipetted into the 24ng/ml tube. It was vortex mixed. Five hundred µl from the 24 ng/ml tube were pipetted into the 12 ng/ml tube. It was vortex mixed again and doubling dilution was repeated successively with the remaining tubes.

4.3.3 Assay protocol

Using the assay microtitre plate, 100 µl of assay buffer were pipetted in the zero standard wells and 100 µl of each standard into the appropriate wells in duplicate using a clean polypropylene pipette tip each time. Then, 100 µl of each of the unknown samples was pipetted in each well in duplicate (see fig 4.1). The plate was covered with the lid and incubated at 20-27°C for 2 hr. The wells were aspirated and washed 4 times with wash buffer ensuring that the wells were completely filled and emptied at

each wash by blotting the plate on tissue paper. One hundred µl of peroxidise antibody conjugate was then incubated for 1hr at room temperature.

Once again, the wells were aspirated and washed 4 times with wash buffer ensuring that the wells were completely filled and emptied at each wash by blotting the plate on tissue paper.

One hundred µl of TMB were dispensed in each well and the plate was incubated for 30 minutes at room temperature (20-27°C). The reaction was stopped adding an additional 100 µl of 1M sulphuric acid into the wells and the plate was read at 450nm within the next 30 min (yellow colour). The standard curve was obtained by plotting the known concentration of each standard with the absorption measured by the plate reader spectophotometer. The standard curve was used to calculate the concentration of MMP2 in the study samples.

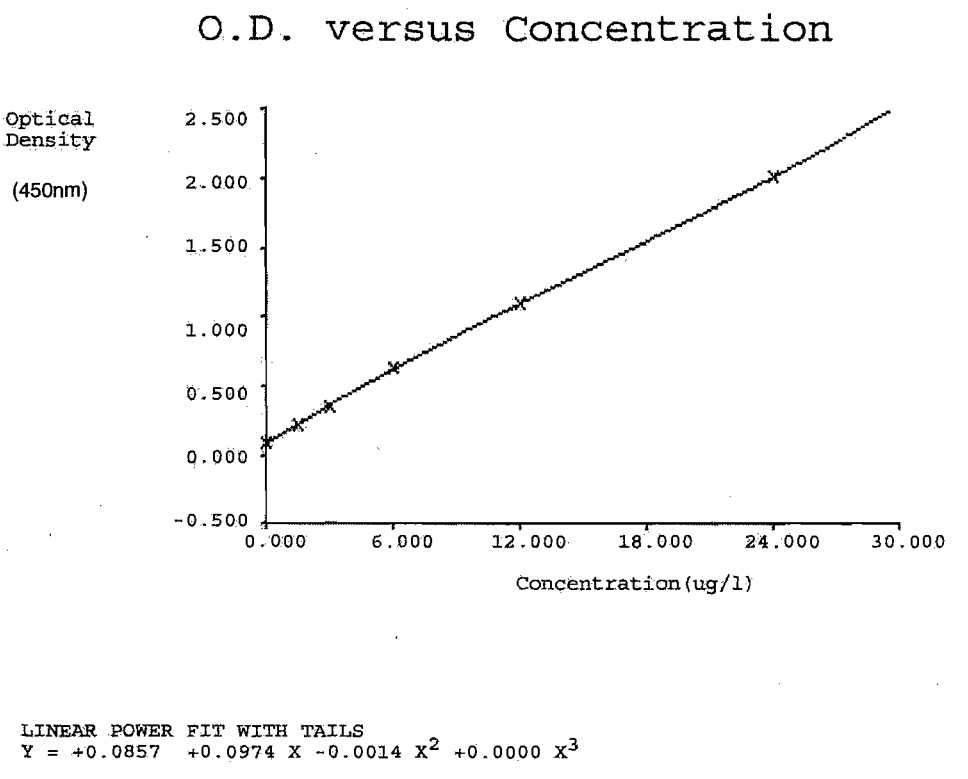


Figure 4.3 Standard curve of the MMP2 ELISA assay

4.3.4 Reproducibility

Within the assay

The within the assay precision for duplicate was calculated by measuring controls in the assay. The results were:

Controls	Mean +/- SD	% CV	n
A	2.91 +/- 0.15	5.3	12
B	5.93 +/- 0.37	6.3	12
C	11.0 +/- 0.59	5.4	12

Table 4.1 Reproducibility within assay precision (mean values as ng/ml equiv. to µg/l) for MMP2 ELISA assay

Between assay

The between assay precision was assessed by repeat measurement of the same sample in successive assays. The results were:

Controls	Mean +/- SD	% CV	n
A	2.44 +/- 0.30	12.2	12
B	5.14 +/- 0.49	9.6	12
C	9.97 +/- 0.83	8.3	12

Table 4.2 Reproducibility between assay precision (mean values as ng/ml) for MMP2 ELISA assay

4.3.5 Specificity

The assay recognises, as explained before, the precursor form of MMP2 (pro-MMP2), free and complexed with TIMP2. It does not recognise active form of MMP2.

The assay does not cross react with MMP1, MMP3, MMP7, MMP8, MMP9 and MT1-MMP.

4.4 Matrix metalloproteinase-2 Activity ELISA Assay

The MMP2 Biotrak Activity Assay System from Amersham Biosciences measures either endogenous active MMP-2(naturally occurring) or total active MMP-2 (following activation using APMA. Standards and samples are incubated in microtitres wells precoated with anti-MMP2 antibody. Any MMP2 present will be bound to the wells and other components of the sample are removed by washing and aspiration. Either the endogenous levels of the free active MMP-2 or the total levels of free MMP2 in a sample can be detected. In order to measure the total MMP2, any bound MMP2 in its pro form is activated using p-aminophenylmercuric acetate (APMA). The standard is pro MMP2 which is activated in parallel for both types of samples. Active MMP2 is detected without APMA treatment. The resultant colour is read at 405nm in a microtitre plate reader. The concentration of the active MMP2 in a sample is determined by interpolation from the standard curve.

Values obtained when APMA was used were well above the middle part of the standard curve. Therefore much higher dilutions of the samples were needed to bring the results to the middle of the standard curve. Dilutions were difficult to obtain and reproduce and therefore this assay was only used to measure active MMP2. Total MMP2 was measured, when needed (see chapter 7), using the assay described previously in this chapter.

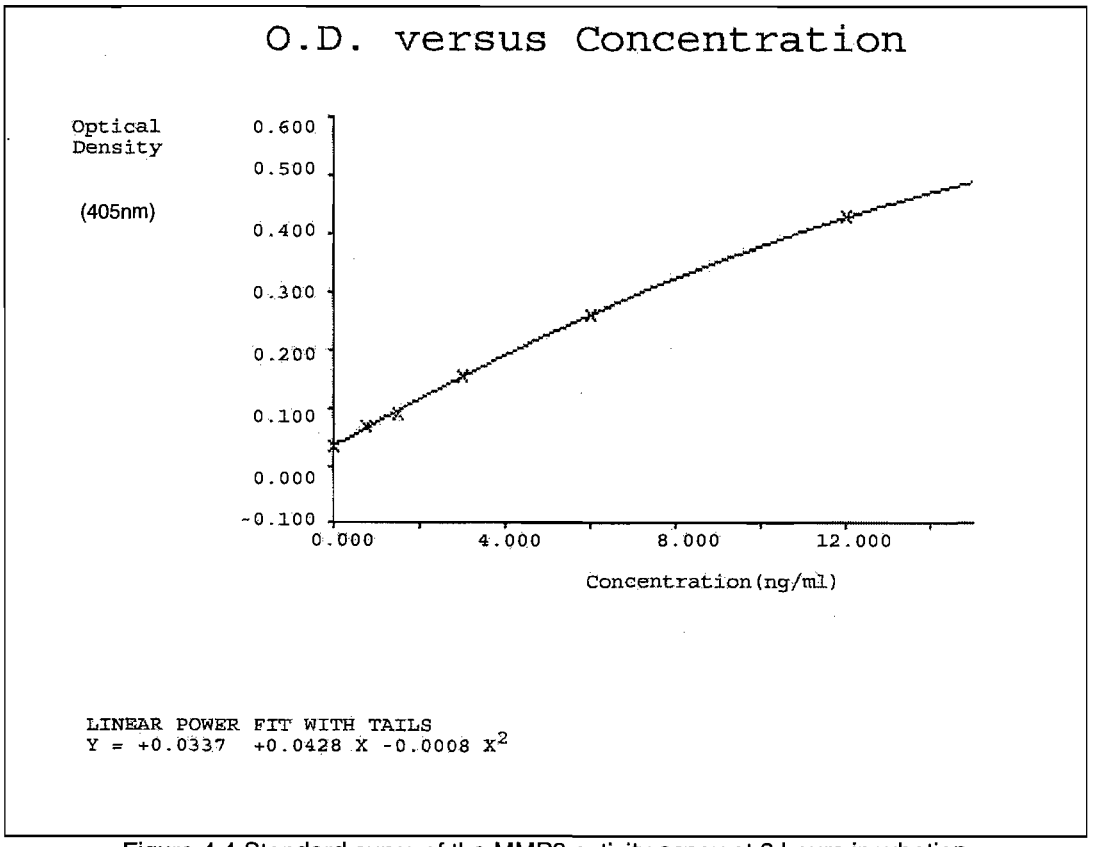


Figure 4.4 Standard curve of the MMP2 activity assay at 3 hours incubation

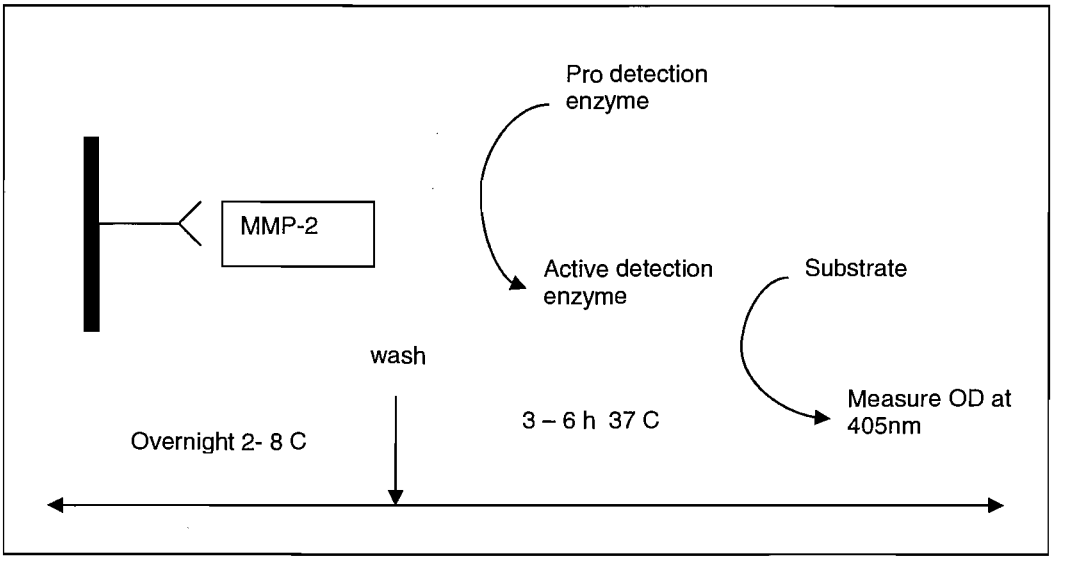


Figure 4.5 Protocol for MMP-2 activity assay measuring endogenous active MMP-2

4.4.1 Preparation of the reagents

Assay buffer

The assay buffer supplied was transferred to a 100 ml measuring cylinder by repeated washing with distilled water. The final volume was adjusted to 100 ml with distilled water and mixed thoroughly.

Standard

One ml of assay buffer was added to the vial containing the standard. It was gently mixed until the contents were completely dissolved. It was stored at 2-8 °C.

Wash Buffer

The wash buffer supplied was transferred to a 500 ml cylinder by repeated washing with distilled water. The final volume was adjusted to 500 ml with distilled water and mixed thoroughly. It was stored at room temperature.

p-Aminophenylmercuric acetate (APMA)

One ml of fresh dimethylsulphoxide (DMSO) was added to the bottle provided containing APMA. It was vortexed until all the powder was in solution. The result was the concentrated 1M solution. Five µl of this solution (1M) was added to a vial containing 10 ml of room temperature assay buffer and it was vortex mixed. This final solution is the ready to use solution (0.5mM).

Detection enzyme

The bottle containing the detection enzyme provided by the assay kit was thawed and stored in ice until use.

Substrate

2.6 ml of assay buffer was added to the bottle containing the substrate. It was gently mixed until the contents were completely dissolved. It was stored on ice until used.

Detection reagent

This reagent was prepared only immediately prior to addition to the wells. Equal volumes of detection enzyme and substrate were mixed in a polypropylene tube according to the number of wells required. Fifty µl of detection reagent is needed for each well which consist of 25 µl of enzyme and 25 µl of substrate.

4.4.2 Preparations of the standards

Five polypropylene tubes for 0.75, 1.5,3,6 and 12ng/ml were labelled. Five hundred µl of assay buffer was added into each tube. Five hundred µl of the stock standard (24ng/ml) were pipetted into the 12ng/ml tube. It was vortex mixed and 500 µl were removed from the 12 ng/ml tube and added to the 6ng/ml tube. It was vortex mixed and the double dilution was repeated successively with the remaining tubes. The result would be 500 µl aliquots of 5 standards.

4.4.3 Assay protocol

There are different protocols according to the endogenous MMP2 levels of the tissue analysed. These protocols were tested with a small number of our samples and in view of the nature of the tissue the assay protocol followed was that corresponding to the high endogenous levels.

The microtitre plate was prepared by pipetting 100µl of assay buffer into the zero standard wells. One hundred µl of each standard were pipetted into the appropriate wells. One hundred µl of the unknown samples were added into the rest of the wells in duplicate as shown in the figure 4.1. A clean polypropylene pipette tip was used for each standard and sample. The plate was incubated at 2-8°C overnight.

The wells were aspirated and washed four times following incubation. Wells were completely filled and emptied each time ensuring adequate wash. Any residual volume was removed by blotting the microtitre plate on tissue paper. Fifty µl of APMA solution were added into the wells containing samples where the total (pro and active) MMP2 activity is to be measured. Fifty µl of assay buffer were added to the samples where only the active MMP2 was to be measured. 50µl of detection reagent were dispensed into each of the wells. The microtitre plate was shaken for 20 seconds and read at 405nm to obtain t_0 (time zero) value.

MMP2 activity was directly proportional to the generation of colour through the cleavage of the S-2444 peptide substrate which can be represented by the rate of

change of absorbance at 405nm. The absorbance change was linear to the square of the duration of the incubation rate. The standard curve obtained was a parabolic curve.

The final data was multiplied by 1000 so as to be able to plot whole numbers on the graph. The data was expressed as:

$$\delta \ Abs_{450}/h^2 * 1000$$

The data obtained at time 0 (t0) was used as a reference point on which the activity rate calculations are based. The absorbance for the standards at t0 was the same for those in the blank. The microtitre plate was then incubated for 3 hours. After being shaken for 20 seconds it was read at 405nm.

Rate of change of MMP-2 activity should be expressed as:

$$\frac{Abs_{t=3} - Abs_{t=0}}{h^2} \quad X\ 1000$$

A standard curve was generated by plotting Absorbance 405/h2 *1000 (y axis) against ng/ml standard (x axis). The concentration of active MMP-2 of the unknown samples was obtained from the standard curve. As the samples were diluted in ¼ to obtain values in the mid zone of the standard curve, then results were multiplied by 4 to obtain the total concentration.

4.4.4 Reproducibility

Within the assay

The within the assay precision for duplicate determination was calculated by measuring controls in the assay. The results were:

Controls	Mean +/- SD	% CV	n
A	4.75 +/- 0.33	7.0	10
B	9.54 +/- 0.52	5.4	10
C	15.63 +/- 0.68	4.4	10

Table 4.3 Reproducibility within assay precision (mean values as ng/ml) for MMP2 ELISA activity assay

Between assays

The between assays precision was assessed by repeat measurement of the same sample in successive assays. The results were:

Controls	Mean +/- SD	% CV	n
A	2.60 +/- 0.44	16.9	12
B	5.48 +/- 0.98	17.9	12
C	9.49 +/- 1.76	18.5	12

Table 4.4 Reproducibility between assay precision (mean values as ng/ml) for MMP2 ELISA activity assay

4.4.5 Specificity

The assay recognises the pro and active forms of MMP2. It does not cross react with other MMPs and TIMPs. Both pro and active MMP2/TIMP2 complexes as well as active MMP2/TIMP1 complexes have shown a degree of cross reactivity in the assay, 8.6 % and 21% respectively.

4.5 Tissue inhibitor of metalloproteinases-2 ELISA Assay

This TIMP2 human Boitrak ELISA System from Amersham biosciences is a two sites based ELISA assay. A peroxidase labelled Fab antibody to TIMP2 is added to standards and samples and the mixture is added to the wells of the microtitre plate which are coated with an anti-TIMP2 antibody. TIMP2 bound to the peroxidase will become bound to the anti-TIMP2 antibody and after washing away other components of the sample the amount of peroxidase is determined by the addition of TMB substrate. The reaction is stopped by adding sulphuric acid and read at 450 nm. The concentration of TIMP2 in a sample is determined by interpolation of the standard curve.

The assay recognizes total TIMP2 (free form and complexes with the active form of MMPs). There is not recognition of the complex form by TIMP2 and pro-MMP2.

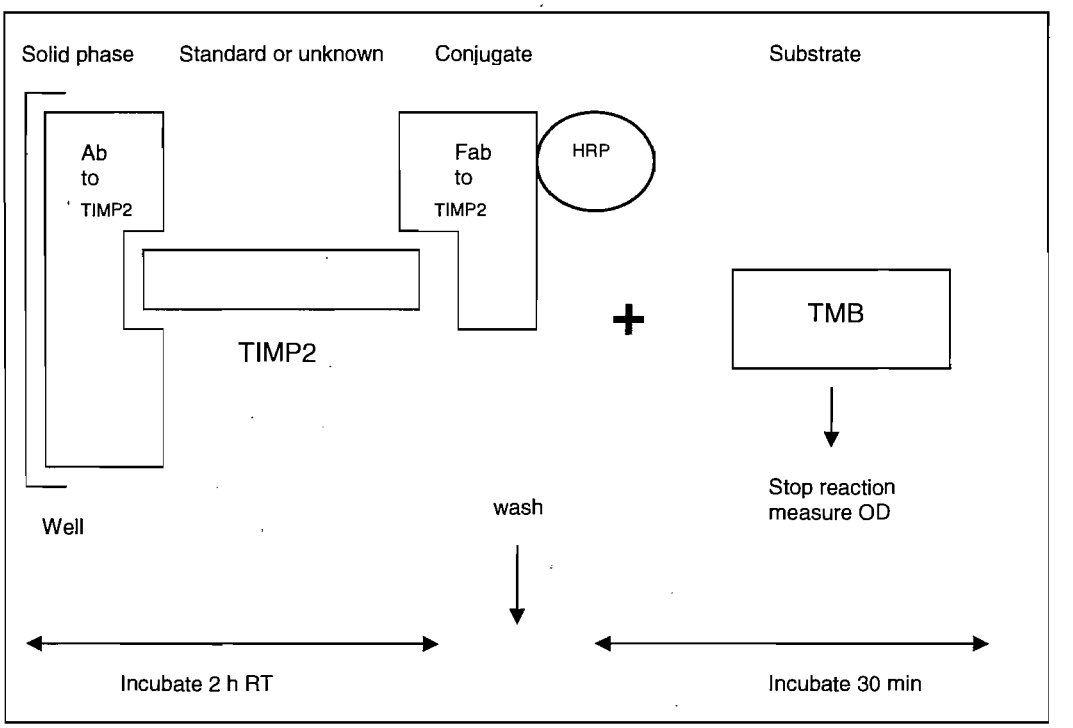


Figure 4.6 TIMP-2 ELISA assay design

4.5.1 Preparation of the reagents

Assay buffer

The contents of the assay buffer were transferred to a 100 ml cylinder by repeated washing with distilled water until obtained a total volume of 100 ml.

Standards

The standard stock was mixed with 1 ml of distilled water until complete dissolution.

Peroxidase conjugate

Twelve ml of distilled water were added to the provided peroxidase and gently mixed until it was completely dissolved.

Wash buffer

The content of the bottle was transferred to a cylinder and distilled water was added until obtaining a total volume of 500 ml. It was mixed thoroughly.

4.5.2 Preparation of the standards

Six tubes of polypropylene were labelled as S0 (0 ng/ml), S1 (8 ng/ml), S2 (16 ng/ml), S3 (32 ng/ml), S4 (64 ng/ml) and S5 (128 ng/ml). Each tube was filled with 500 µl of assay buffer. Five hundred µl of standard stock was added to S5 tube. It was vortex mixed and 500 µl of the solution were extracted and added to S4 tube. This doubling dilution step was repeated successively with the remaining tubes until S1. No standard stock was added to S0.

4.5.3 Assay protocol

Forty eight polypropylene tubes were labelled with all the standards and the unknown samples code. One hundred µl of the each standard were pipetted into the corresponding labelled tube. One hundred µl of each of the unknown samples were added to the appropriate tube. One hundred µl of peroxidase conjugate was added into all the tubes and vortex mixed. Ninety five µl of each mixture were pipetted into the appropriate wells in duplicate (see fig 4.1). After two hour incubation period the wells

were washed carefully with the assay wash buffer and the plate was blotted into a tissue paper to remove any remaining solution. This procedure was repeated 4 times. Immediately after 100µl of TMB substrate was added to the wells and left for 30 min at room temperature. The reaction was stopped by adding 100µl of sulphuric acid into all the wells. The plate then was read at 450nm within 30 min. The standard curve obtained was used to calculate the value of the samples from the study.

4.5.4 Reproducibility

Within the assay

It was calculated by measuring controls in the assay. The results were:

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

Table 4.5 Reproducibility within assay precision (mean values as ng/ml) for the TIMP2 ELISA assay

Between assays

It was calculated by measuring the same sample in successive assays. The results were:

Controls	Mean +/- SD	% CV	n
A	16.0 +/- 0.94	5.9	12
B	29.5 +/- 1.76	6.0	12
C	50.1 +/- 1.25	2.5	12

Table 4.6.Reproducubility within assay precision (mean values as ng/ml) for TIMP2 ELISA assay

4.5.5 Specificity

The assay recognises free TIMP2 and that complexed with the active forms of MMPs). It does not cross react when TIMP2 and that complexed with the precursor of MMP2 (pro-MMP2). The assay does not cross reacts with TIMP1 or TIMP3.

METHODOLOGY

Chapter 5

Molecular biology

Molecular biology

5.1 Collagen XVII

As described in the Introduction (chapter 1), collagen XVII is a transmembrane protein involved in cell adhesion of the epithelial cell to the base membrane. It has also regulatory roles and the molecule of collagen XVII has an intracellular portion and an extracellular part which is shed by enzymes from the desintegrin and matrix metalloproteinases family (ADAMs).

The assessment of collagen XVII protein is extremely difficult due to the shedding of the molecule from the extracellular matrix and the production of autoantibodies. Therefore measurement of mRNA expression of collagen XVII is an ideal way of assessing how the skin is reacting to its requirement for collagen XVII.

5.2 Preparation of the samples

Samples were kept on dry ice to avoid thawing while the cutting stage was taking place. Cutting of the samples was performed on dry ice using a Petri dish, small tissue holder and the blade which were cooled on dry ice for a few minutes before use.

Samples were cut and weighed using a highly sensitive balance and then they were stored individually in cooled labelled containers. Samples were then ready for the RNA extraction and were kept in the freezer at -80°C until required.

Samples weighing less than 28 or more than 32 mg were used. Any sample that thawed during the procedure of cutting was discarded.

5.3 RNA extraction and quantification of RNA

The SV Total RNA Isolation System (Cat # Z3100, Lot # 188423) from Promega was the method used for RNA isolation from the tissues of interest. The successful isolation of intact RNA requires four essential steps: effective disruption of tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNAase) activity and removal of contaminating DNA and proteins. The most important step is the inactivation of endogenous RNAases that are released from the membrane-bound organelles upon cell disruption to stop degradation of RNA.

Guanidine thiocynate (GTC) and beta-mercaptoethanol were used to inactivate the ribonucleases present in the cell extracts. GTC acts to disrupt nucleoprotein complexes, allowing the RNA to be released into the solution and isolated free of protein.

Samples were homogenised with the lysis buffer. The amount of lysis buffer used was proportional to the sample weight.

Formula for calculating lysis buffer:

Using 30 mg of tissue / 175 µl of buffer lysis

Then total lysis buffer (µl) = (sample weight x 175 µl) / 30

Homogenisation was performed using a Labour Technik T8.01 Ultra-Turrax homogenizer by IKA-WERKE, GMBH & Co.KG, (Staufen, Germany). The samples were homogenised for 30 sec and then cooled on ice. Samples were kept on ice to avoid RNAase activation and gloves were used to avoid contact with the RNAases present on the fingers and hands.

Three hundred and fifty ml of SV RNA dilution buffer were added to each sample and mixed. The tubes were heated at 70° C for 3 min and then centrifuged. The supernatant was extracted and transferred to a clean tube. The pellet was discarded. Two hundred ml of 95% ethanol were added to each tube and mixed. Then it was transferred to the spin basket assembly and centrifuged for 1 min. Each spin basket assembly consists of a Spin basket and a 2 ml collection tube. The RNA is selectively precipitated with the ethanol and bound to the silica surface of the glass fibres found in the spin basket. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favouring absorption of nucleic acids to the silica.

Six hundred ml of SV RNA Wash Solution were added to each tube and centrifugation was performed. DNase treatment was performed by adding 50µl of DNase mix. The DNase mix solution was prepared at the time of the assay mixing 40 µl of beta-mercaptoethanol, 5 µl of Mn Cl_2 and 5 µl of DNase I per sample. These quantities

would be multiplied by the number of samples being analysed each time to obtain enough solution. RNase free DNase I was applied to the silica membrane to digest contaminating genomic DNA. After 15 min incubation at room temperature, 200µl of DNase stop solution was added to each sample and they were centrifuged. Six hundred ml of SV RNA wash solution was added and samples were centrifuged. The eluant was discarded. A further 250 µl of RNA wash solution was added and centrifugation was performed again. The eluant was discarded. These last two washing steps purify further the total RNA bound from contaminating salts, proteins and cellular impurities. Finally the total RNA was eluted from the membrane by adding 100µl of Nuclease free water. It was kept at -80 °C until used for reverse transcription.

Quantification of RNA

To confirm the presence of RNA in the samples and verify that extraction had been done correctly, RNA was quantified prior to starting reverse transcription.

Ten ml of the RNA sample was added to 0.5 ml of purified water. A spectrophotometer (Jenway 6505 UV/vis (UK)) was used. RNA absorbs at 260 nm while proteins absorb at 280nm. The spectrophotometer gives the absorbance (A) at 260nm and ratio A for 260/280 nm. The ratio between the 260 absorbance and the 280 absorbance should be from1.5 to 2. The purity of RNA was also checked periodically by running electrophoresis to check clean bands of ribosomal RNA (this is more readily viewed than mRNA) were obtained and not smeared up a gel when degradation of RNA occurs.

5.4 Reverse transcription

A reverse transcriptase is a DNA polymerase enzyme that copies single-stranded RNA into a copy of complimentary DNA (cDNA). Normal transcription involves the synthesis of RNA from DNA, hence reverse transcription is the reverse of this, as it synthesizes DNA from RNA, yielding cDNA.

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantification currently available. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller amounts.

The reverse transcriptase reaction was performed in two steps:

First step: 2 µl of extracted RNA were added to 0.8µl of random primers (500 µg/ml, Promega) and 12.2 µl of purified water (Sample 1). The mixture was heated at 70 °C for 5 min and cooled by placing on ice for 3 min.

Second step: A master mix containing the following amounts of reagents for each sample was made up.

- RT buffer	5 µl
- dNTPs (PCR mix 10mM)*	1.25 µl
- RNAsin (40U/ µl)	0.625 µl

- M-MLV transcriptase (200U/ µl) 1 µl
- Ultra- pure water 2.125 µl

* 10mM each of: dATP, dCTP, dGTP, dTTP; Promega.

A final volume of 10 µl was added to each Sample 1 from the first step. The total was mixed, spun down and incubated for one hour at 37 °C, then 10 minutes at 42 °C and 10 minutes at 75°C.

5.5 Polymerase chain reaction (PCR)

5.5.1 Design of primers and probes

The primers were short sequences of oligonucleotides complimentary to specific areas of the mRNA sequence of collagen XVII. They encompassed the specific area of the collagen XVII mRNA to be amplified in the PCR. These primers were designed for our study using Primer Express software and the collagen XVII mRNA sequence (see below). The sequences corresponding to exons and introns present in the genome were identified. The exons code for protein while introns have no protein code but can be involved in mRNA expression, although when the mRNA is fully synthesized the intron areas are spliced out of the mRNA. Therefore, if there is genomic DNA contamination then there will be intron information present in the sample while only exon information will be present in pure mRNA samples.

If one of the primers in a PCR reaction includes the end of an exon and the beginning of the next exon then only mRNA will be amplified and any contaminating DNA will be ignored. In real-time PCR, a probe which corresponds to a middle piece of the PCR amplified product is also designed to react with amplified DNA and generate fluorescence which is proportional to the amount of product. (see below).

The gene for collagen XVII is on chromosome 10 and contains exons and introns.
Primer forward (sequence written from 5' to 3')
...GCCGGCTGGAGAAACAAAG (Blue uppercase below and from positions 318-336)

Primer reverse (sequence written from 5' to 3')
...CTCGTGTGCTTCCAGTTGAGTT (Blue uppercase below from complimentary sequence and in reverse, positions 386-365)

For this primer there is an exon-exon junction at 371 – 372, exon 4 –exon 5, and an intron sequence has been spliced out.

Probe

....CTGACTCATGGCGCAGCGGCTACAT

This is below in red uppercase letters between the primer areas.

The primers and probes were manufactured by Eurogentec Ltd (Winchester Hill Commercial Park, Winchester Hill, Romsey. www.eurogentec.com).

Figure 5.1 Sequence of Homo Sapiens collagen XVII mRNA. Accession number NM_000494 from the NCBI website http://www.ncbi.nlm.nih.gov for collagen XVII

Concentration of collagen XVII in the samples was related to 18s, which is a ribosomal RNA present in the cell and an indirect way of measuring the quantity of RNA in the sample.

Primers and probe for the 18s assay (18s rRNA control kit , FAM-TAMRA, Ref. RT-CKFT-18s) were manufactured by Eurogentec Ltd (Winchester Hill Commercial Park, Winchester Hill, Romsey. www.eurogentec.com).

5.5.2 Optimization of primers

Different concentrations of forward and reverse primers were tested to find the optimal concentration for each one. Ten tubes were labelled with numbers 1 to 10. Tube 10 or NTC would not contain cDNA.

The following was performed:

- Three µl of probe and 50 µl of universal mix were mixed for each reaction.
- 53 µl of the mixture was removed to the DNA free sample or NTC tube.
- 40 µl of cDNA were added to the remaining volume of the mixture.
- 57 µl of the mixture were added to each tube 1 to 9.

The forward, reverse primers and purified water were added following the concentration table shown below:

Tube numbers	1	2	3	4	5	6	7	8	9	10(NTC)
primer ratios	50/50	300/50	900/50	50/300	300/300	900/300	50/900	300/900	900/900	900/900
forward primer	0.5	3	9	0.5	3	9	0.5	3	9	9
reverse primer	0.5	0.5	0.5	3	3	3	9	9	9	9
Water	42	39.5	33.5	39.5	37	31	33.5	31	25	29

Table 5.1 Different dilutions for the optimization of the primers and probes for collagen XVII

An ideal concentration of 300nM/300nM was found to have the lowest Ct (lowest number of cycles before detection of significant amounts of fluorescence) value which it meant this was the ideal reaction to produce the most sensitive reaction

5.5.3 Real-time PCR

For the collagen XVII assay, a master mix was made up according to the number of samples required for analysis. For each sample it contained:

- 3 µl probe (5uM)
- 3 µl forward primer (10uM)
- 3 µl reverse primer (10uM)
- 50 µl of Universal mix
- 37 µl of ultra pure water

Ninety six µl of the mixture were added in a small eppendorf tube and 4ul of cDNA were added to each tube. They were vortex mixed and centrifuged for 10 sec. As each sample was analyzed in duplicate, 45 µl of each sample was dispensed in duplicate into microtitre plate wells.

For the 18s assay (Ref. RT-CKFT-18s, Eurogentec) a different master mix was made up according to the number of samples. For each sample it contained:

- 3 µl of primer mix (10uM)
- 1 µl of probe (6.25 uM)

As for the collagen XVII, 96 µl of the mixture were added to each eppendorf tube containing 4 µl of each cDNA sample which had been diluted to 1/100. After vortex mix, 45 µl of the mixture were dispensed in duplicates in a microtitre plate wells.

The plates containing either collagen XVII assay or 18s assay were then analyzed using real time PCR. Each assay was performed using a standard curve with 7 standards and six controls to check reproducibility.

Real time PCR was performed on an Applied Biosystems ABI Prism ® 7700 Sequence Detection System. The system consists of a 96 well thermal cycler. Real time PCR is a method of detecting quantitatively mRNA. It is superior to traditional RNA as it detects RNA in the exponential phase, not in the plateau phase or end point measurement of accumulated PCR products used by traditional PCR. An oligonucleotide called a TaqMan® Probe is added to the PCR reagent master mix. The probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5' exonuclease activity of the enzyme cleaves the probe. The TaqMan® Probe is designed with a high-energy dye termed a Reporter at the 5'end (usually 6- carboxyfluorescein), and a low-energy molecule termed a Quencher at the 3' end (fluorochrome, 6- carboxy-tetramethyl-rhodamine). When this probe is intact and both fluorescences are in the probe, the quencher molecule stops all fluorescence by the reporter. As Taq polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of the Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification of each cycle is proportional to the amount of product generated in each cycle.

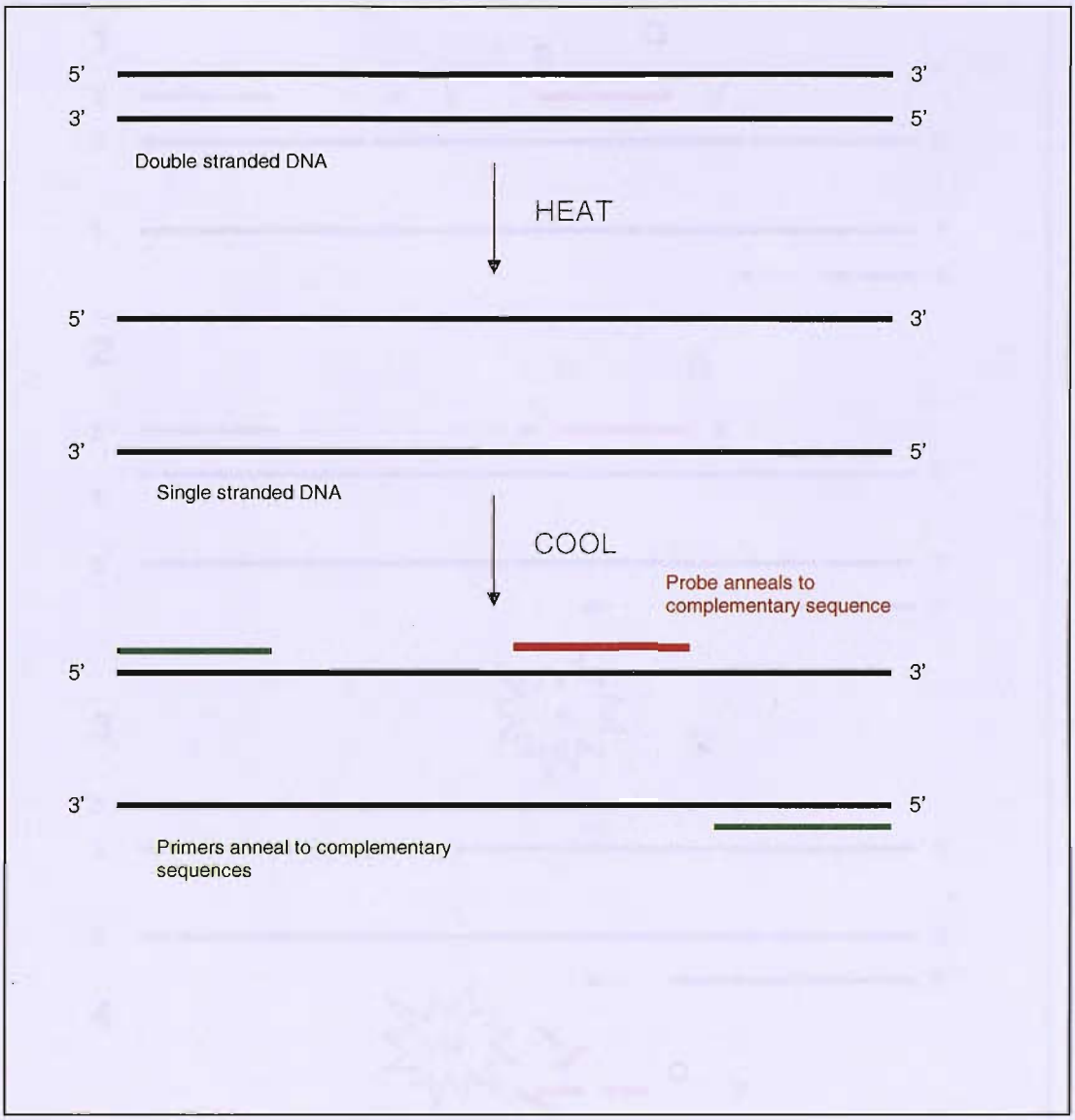
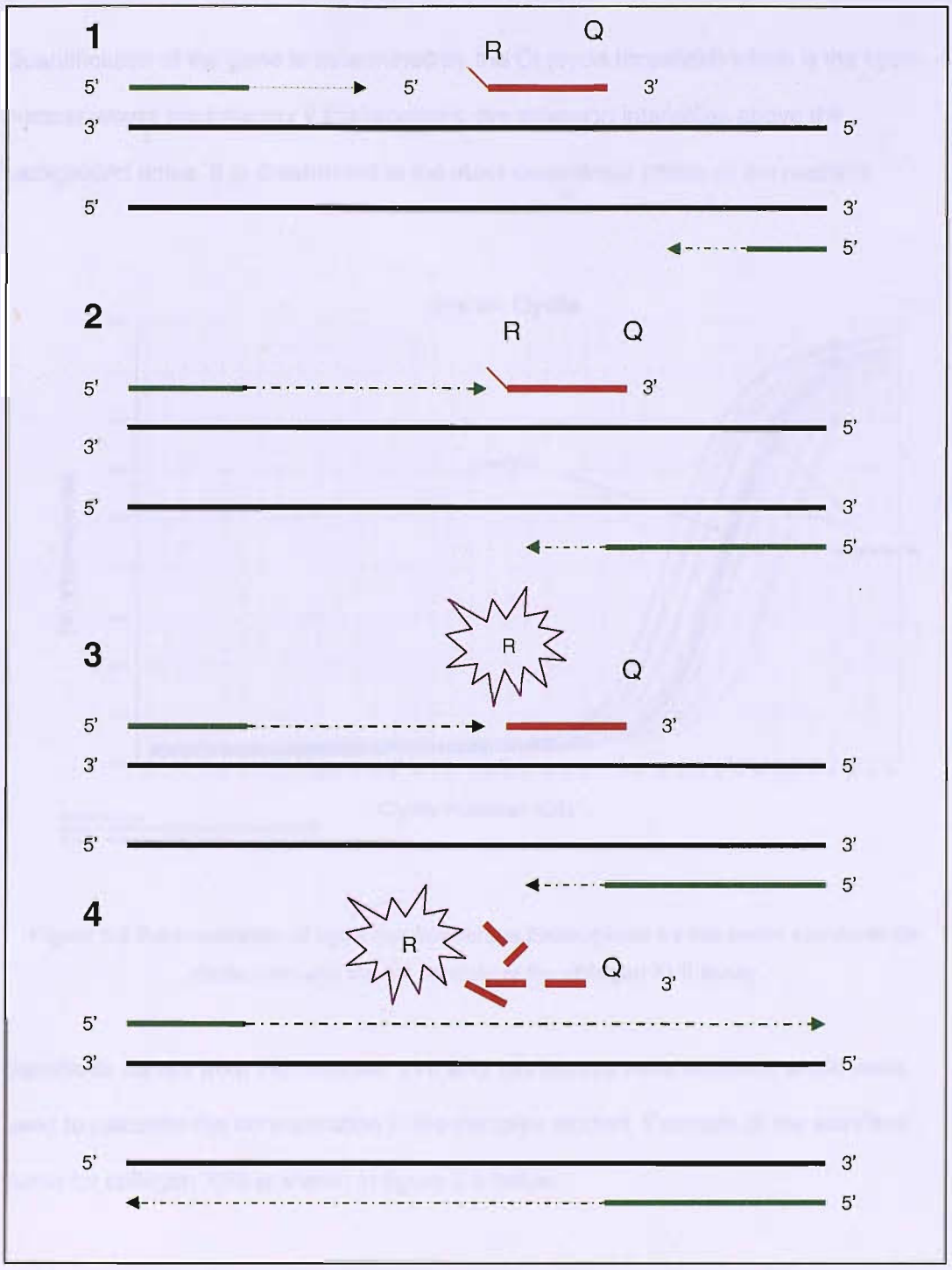


Figure 5.2 Real-time Polymerase chain reaction. Cycles are repeated to amplify the product. Assays were performed below for 40 cycles with denaturation at 95°C for 15sec and annealing and extension at 60°C for 1 min.



.Figure 5.3 Forward and reverse primers are extended with Taq polymerase. 2) As the polymerase extends the primer, the probe is displaced. 3) An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. 4) After release from the reporter dye from the quencher, a fluorescent signal is generated.

Quantification of the gene is determined by the Ct (cycle threshold) which is the cycle number where the intensity if the reporter's dye emission intensifies above the background noise. It is determined at the most exponential phase of the reaction.

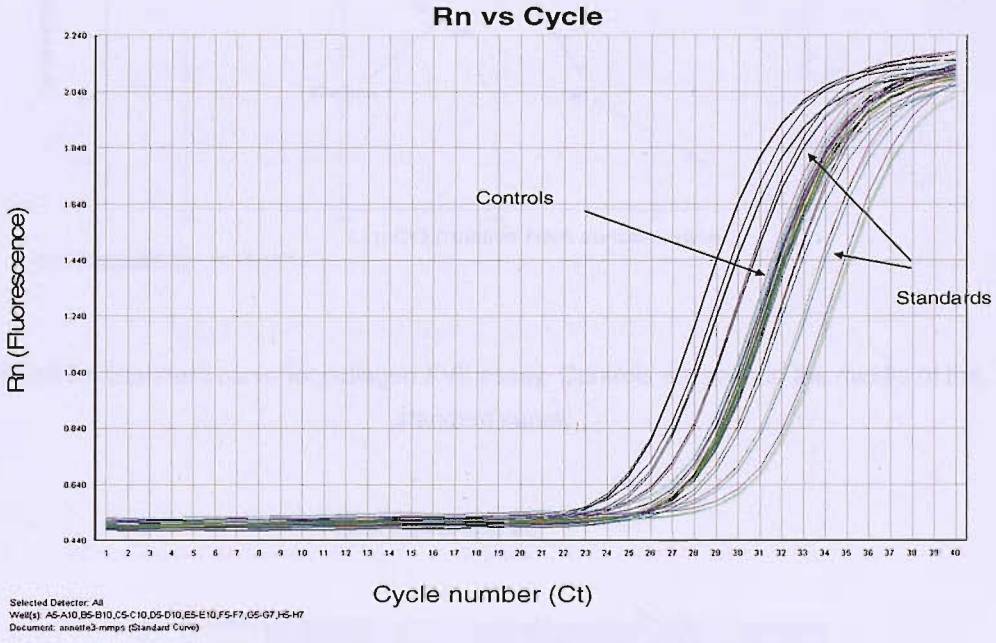


Figure 5.4 Representation of cycle number versus fluorescence for the seven standards (in duplicates) and the six controls of the collagen XVII assay

Standards curves from the collagen XVII and 18s assays were obtained which were used to calculate the concentration in the samples studied. Example of the standard curve for collagen XVII is shown in figure 5.5 below.

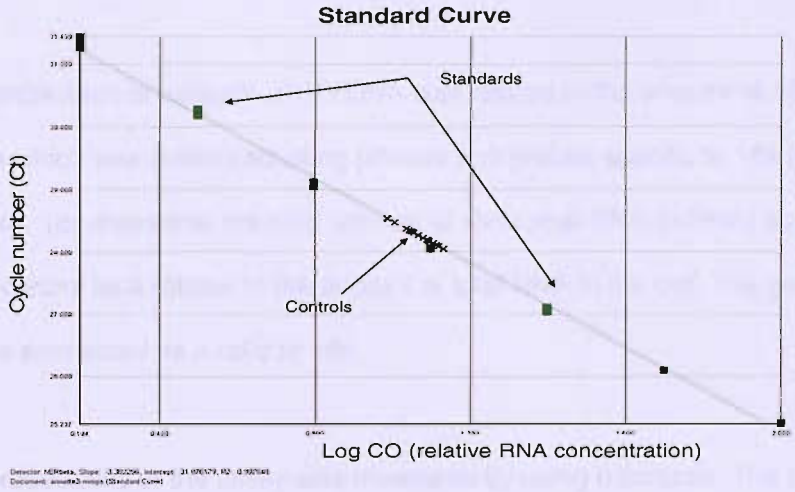


Figure 5.5 Standard curve for collagen XVII assay. Controls are seen in the middle of the standard curve.

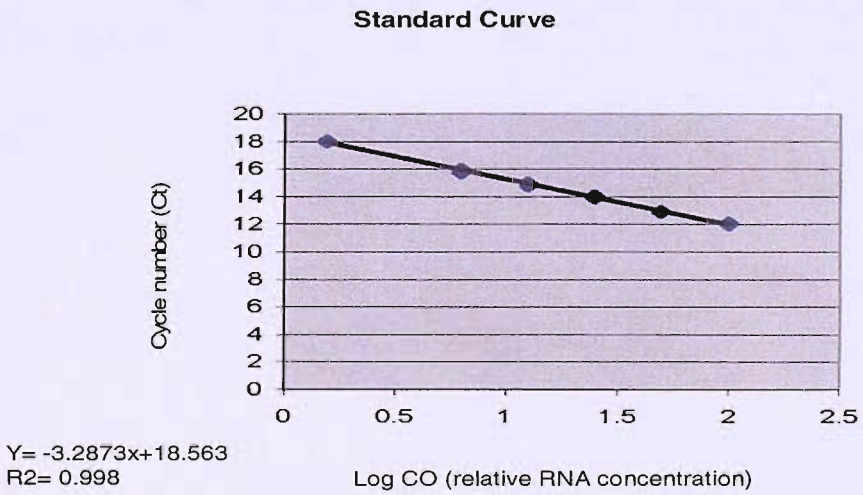


Figure 5.6 Standard curve for the 18s assay

5.6 Quantification of gene expression and reproducibility

The quantification of collagen XVII cDNA was related to the amount of 18s in the samples which was measured using primers and probes specific to 18s (Eurogentec kit as above). 18s measures the total amount of ribosomal RNA (mRNA) so acts as an internal control as it relates to the amount of total RNA in the cell. The gene of collagen XVII was expressed as a ratio to 18s.

The reproducibility of the assay was measured by using 6 controls. The coefficient of variation for the six controls was consistently less than 11%.

METHODOLOGY

Chapter 6

Immunohistochemistry

Immunohistochemistry

Immunohistochemistry combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. Immunohistochemistry makes it possible to visualize the distribution and localization of specific cellular components within a tissue. The immunohistochemistry procedure consists of tissue preparation, antibody incubation and a series of detection reactions. Collagen I and III were studied in our samples using this technique which is described in detail in this chapter.

6.1 Preparation of the tissue samples

After obtaining the sample from paraurethral skin in the operating theatre it was sent to the laboratory to be embedded in to paraffin blocks as described in Chapter 3. Tissue is hardened by replacing the water with paraffin to be able to be cut in very thin layers. The samples were transported to England as paraffin blocks at room temperature.

A microtome with steel blade was used to prepare the sections for histology treatment. Four µm paraffin sections were cut and floated in warm water. Sections were floated onto glass slides coated with aminopropyltriethoxysilane (APES) which provides an adhesive for the tissue to stick to the glass avoiding migration during antigen retrieval (see below). After overnight incubation at 37°C the slides were stored at room

temperature. All the slides were labelled in pencil, coding the samples and keeping the researcher blind to origin during the Image analyses.

6.2 Immunohistochemistry methods

The method used in this project was an antibody conjugated to the enzyme peroxidase that can catalyze a colour-producing reaction. The first step in this immunoperoxidase staining is the binding of the specific (primary) antibody (first layer) which reacts with the tissue antigen and a labelled secondary antibody (second layer) which reacts with the primary antibody. The secondary antibody is raised in another animal against the immunoglobulin type of the primary. This method is more sensitive than the direct one due to the signal amplification through several secondary antibody reactions with the different antigenic sites and the primary antibody. The second layer antibody can be labelled with an enzyme. The reaction is visualized by applying a mixture of substrate and chromogen, which is converted by the conjugated enzyme to produce a colour change. Subsequently the colour is proportional to amount of enzyme which is proportional to antigen of interest.

Any endogenous enzyme of the same type as that used to label the secondary antibody needs to be blocked and therefore endogenous peroxidase activity needed to be blocked in the present assays.

Samples were dewaxed in xylene and rehydrated through graded alcohols to 70% alcohol before staining was started.

Once the sections were rehydrated, the endogenous peroxidases were inhibited with 0.5% hydrogen peroxidise in methanol for 10 minutes. After this time, the sections were washed with TBS for two minutes, three consecutive times.

6.3 Antigen retrieval

After endogenous peroxidases were inhibited, antigen retrieval was carried out to reveal antigens which were masked by earlier formalin fixation. Antigen retrieval appropriate to the antibody was performed, followed by blocking of non-specific avidin-biotin interactions (Vector, Peterborough, UK Avidin –biotin blocking kits. Catalog number SP-2001). The procedure ensures all endogenous biotin, biotin receptors, or avidin binding sites present in tissues are blocked prior to the addition of the labelled avidin reagent. Then, three washings of 2 min each with TBS were performed. After draining the slides, non-specific protein interactions were blocked with a solution of DMEM culture medium containing 20% fetal calf serum and 1% BSA for 20 min.

The primary antibodies were applied on to the slides and incubated overnight at 4 °C. Collagen I was a mouse monoclonal from Abcam (Cambridge, UK) and collagen III a rabbit polyclonal from Cedarlane Laboratories Ltd (Canada). (Monoclonal antibodies are produced from cloned immune cells and often recognise one epitope whereas polyclonal antibodies are heterogeneous mix of antibodies that recognize several epitopes.)

After incubation three washes were performed by leaving the slides for 5 min in TBS three times. Slides were then drained and biotinylated secondary antibodies, against

primary antibodies (Dakocytomation, Ely, UK) were applied for 30 min. In this study secondary antibodies were anti-mouse IgG for the collagen I assay and anti-rabbit IgG for collagen III. Slides were washed three times on TBS for 5 min and drained. Streptavidin biotin-peroxidase complexes (ABC) were applied for 30 min (Dakocytomation, Ely, UK). Slides were washed (3 X 5 minutes) and drained.

(Streptavidin is a tetrameric 60 KD Avidin analogue isolated from the bacterium Streptomyces avidinii. It is capable of binding four molecules of biotin with a very high affinity. This affinity is approximately 106 times greater than that of most antibodies for their antigens and provides very specific detection and amplification of the antigen-antibody binding event.)

A variety of chromogens have been used to localize peroxidase in the tissue sections. The most commonly used have been diaminobenzidine tetrahydrochloride (DAB) DAB produces a reddish brown precipitate in the sections. DAB is insoluble in alcohol and clearing agent allowing sections to be permanently mounted. Other commonly chromogens used are 3 amino-9-ethyl carbazole (AEC) which produces a red reaction and TMB which produces a blue precipitate.

The substrate used in this study was diamonobenzidine to yield a brown reaction product. It was applied for 5 min, rinsed in TBS and then washed with running tap water for 5 min.

Sections were counterstained with Mayers haematoxylin (blue), dehydrated through graded alcohols, cleared in xylene and mounted in DPX. They were stored at room temperature until analysis.

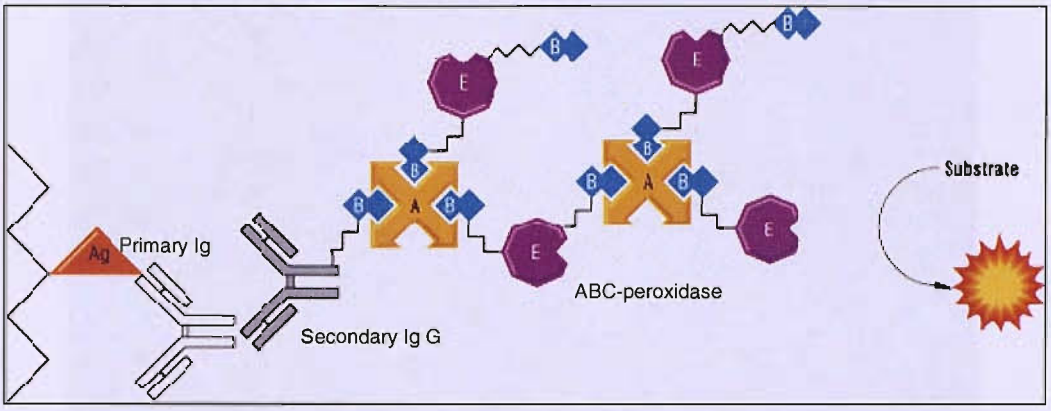


Figure 6.1 Three layer immunohistochemistry using the avidin-biotin complex (ABC) method

Each sample was stained for collagen I and collagen III. Both of those samples had a negative control. These controls lacked primary antibody, secondary antibody and streptavidin-enzyme conjugates.

Examples of negative control, collagen I and collagen III staining for the four groups are shown bellow:

Figure 6.2 Negative control for collagen I staining

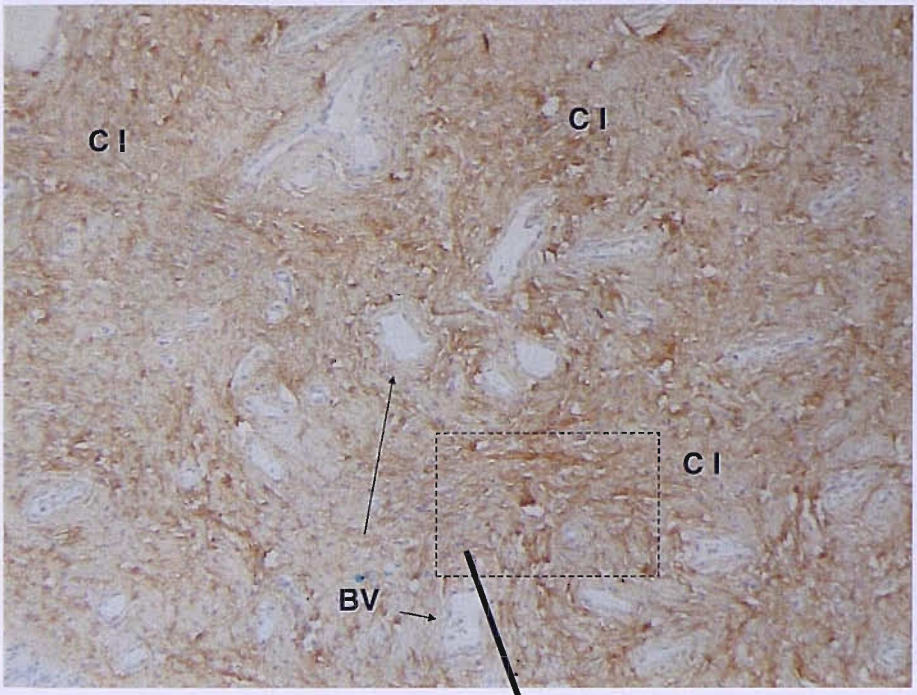


Figure 6.3 Collagen I (C I) staining for WC, seen as brown staining. Blood vessels (BV) do not stain. Microscope magnification was 10X

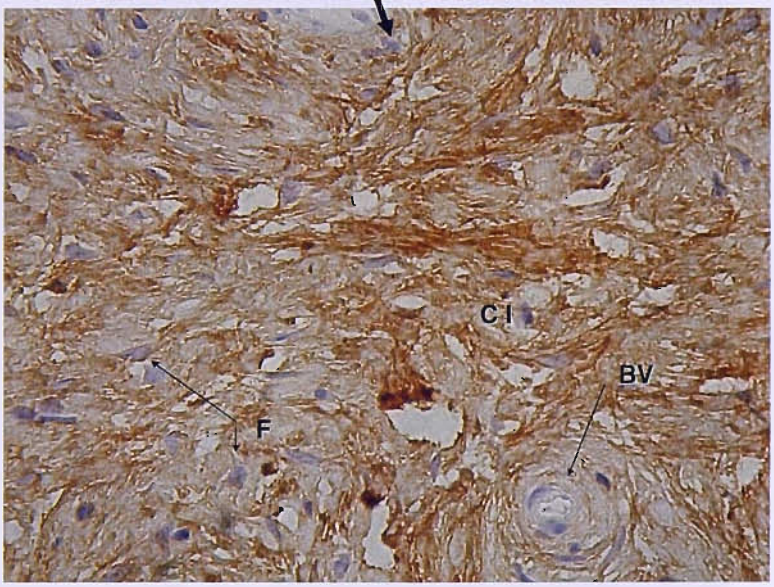


Figure 6.4 Collagen I staining in WC. Microscope magnification 40X from highlighted area in figure 6.3

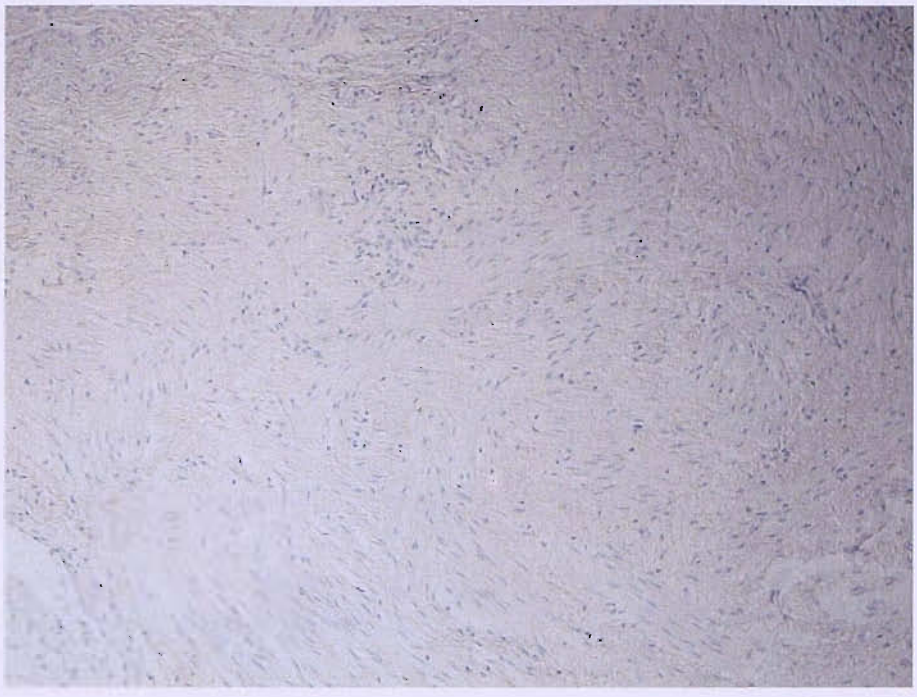


Figure 6.5 Example of negative control for collagen III. Microscope magnification is 10X

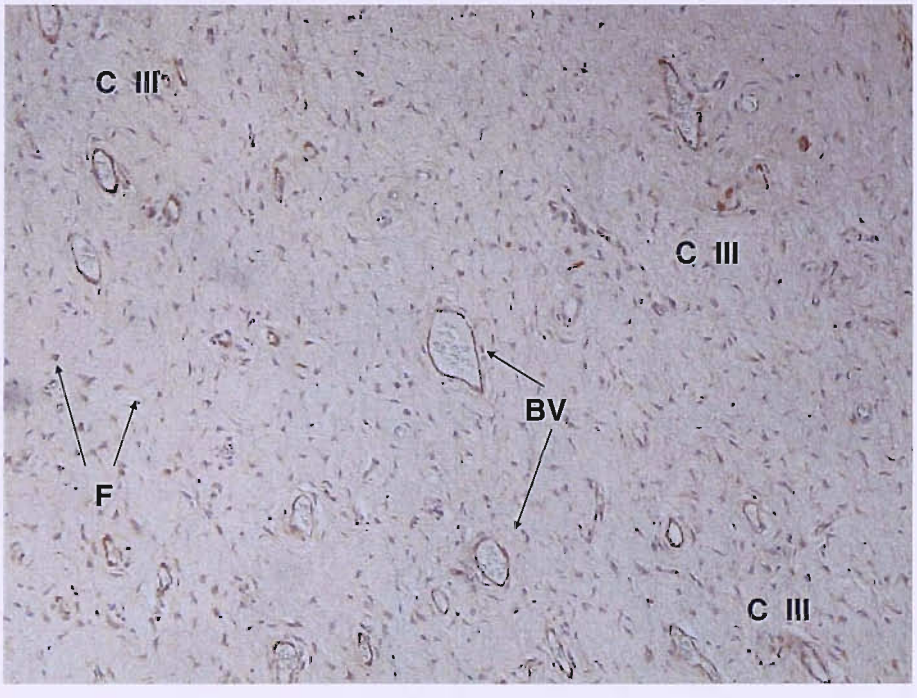


Figure 6.6 Collagen III staining for WC seen in brown. Fibroblast (F) and blood vessels (BV) are identified. Microscope magnification is 10X

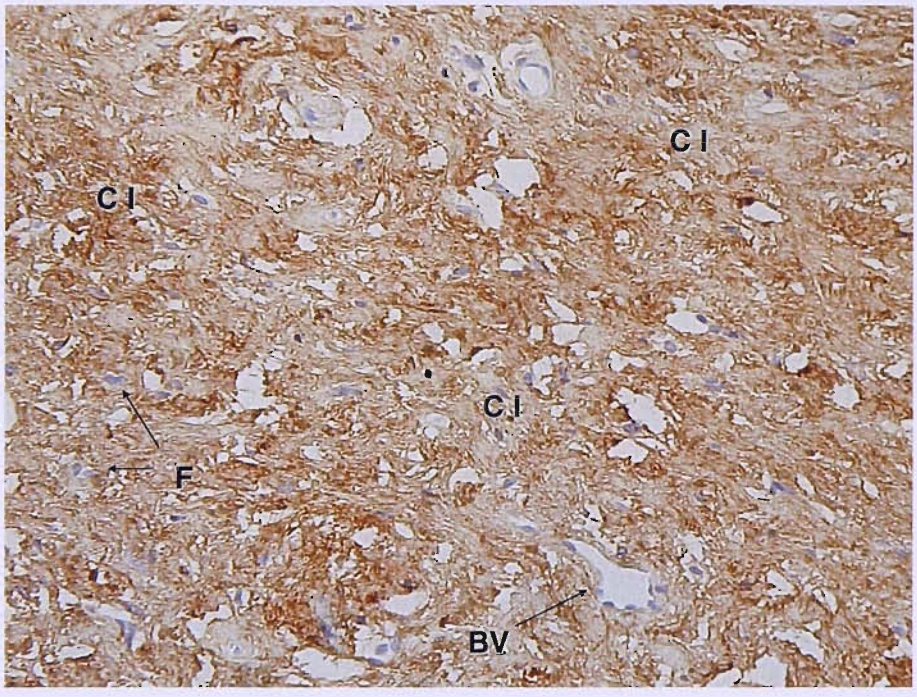


Figure 6.7 Collagen I staining in WSI group. Microscope magnification is 20X

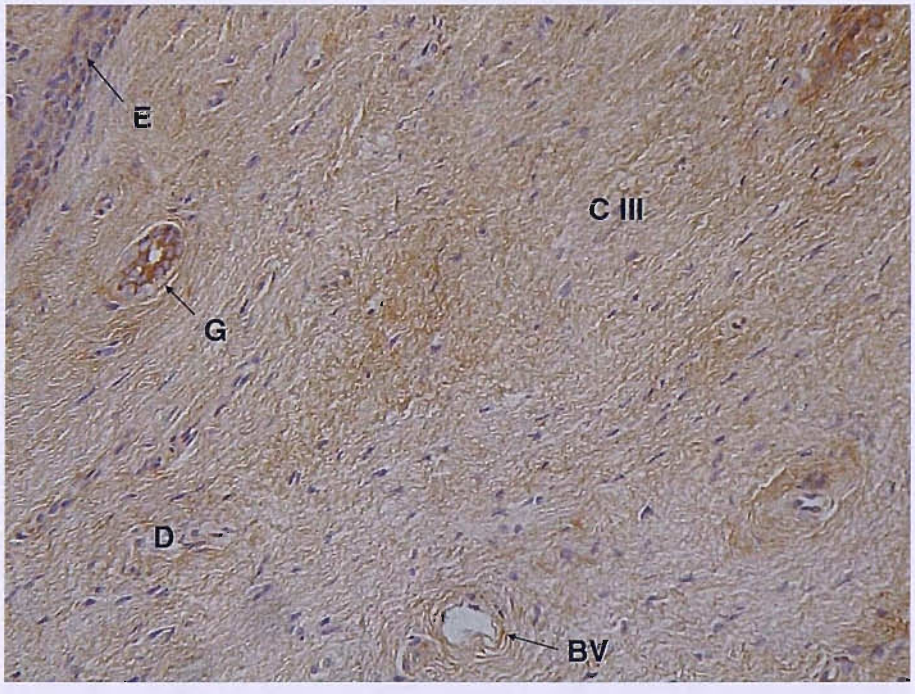


Figure 6.8 Collagen IIII staining for WSI group. Collagen III (CIII), blood vessels (BV) and a gland (G) are seen in the dermis (D). The epidermis (E) was excluded from analysis. Microscope magnification is10X

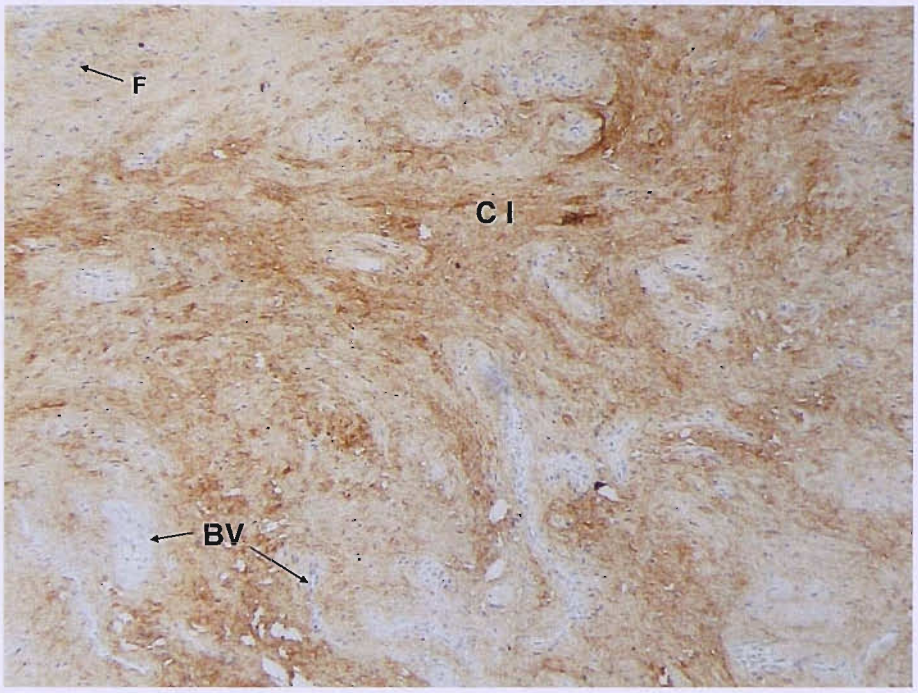


Figure 6.9 Collagen I staining for BC group, brown staining. Fibroblast (F) and blood vessels (BV) are also seen. Microscope magnification 10X

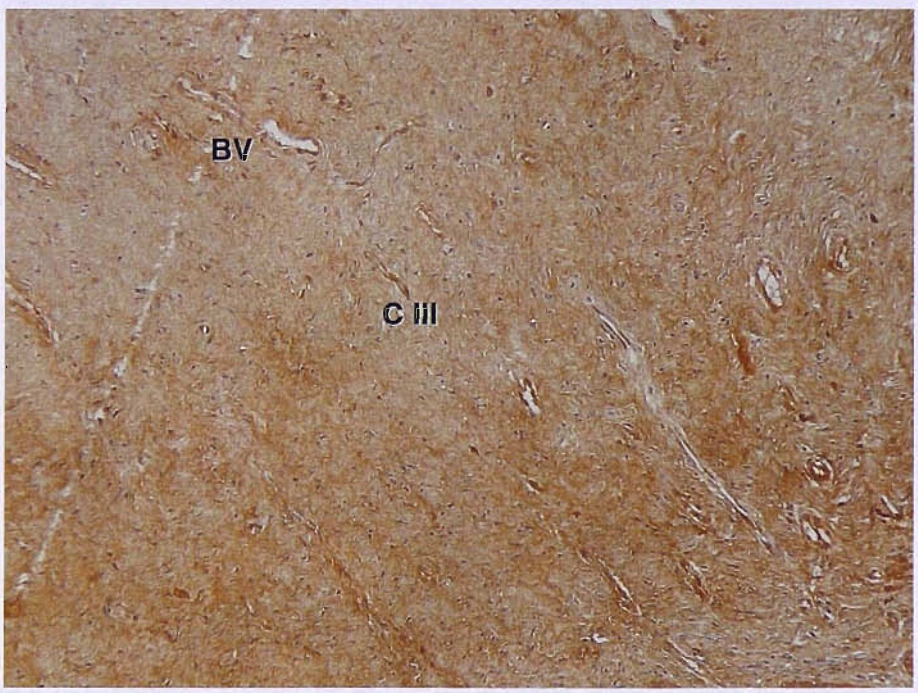


Figure 6.10 Collagen III staining for BC group. Microscope magnification 10X

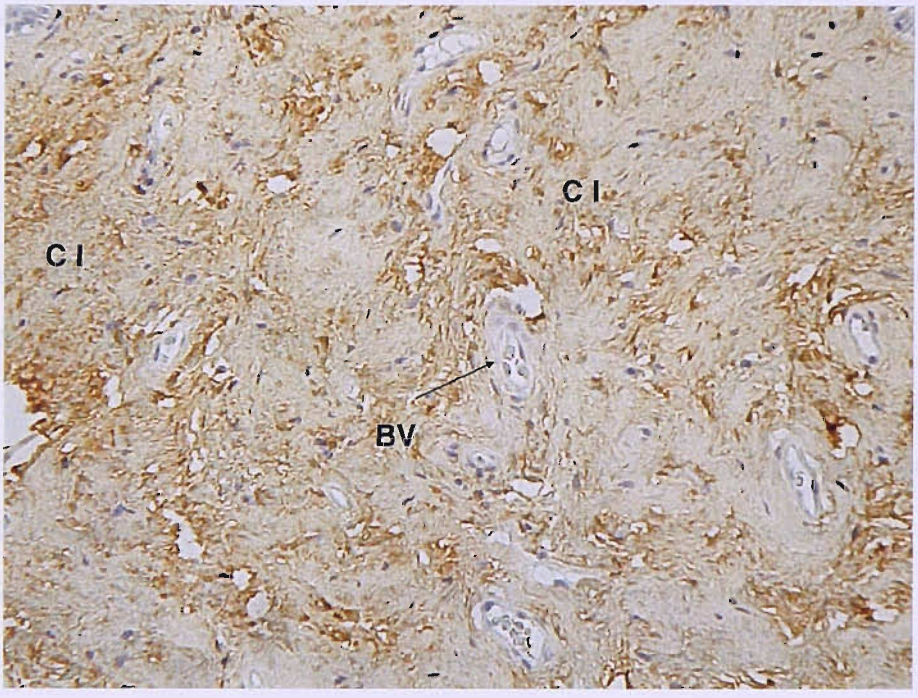


Figure 6.11 Collagen I staining for BSI. Microscope magnification 20X

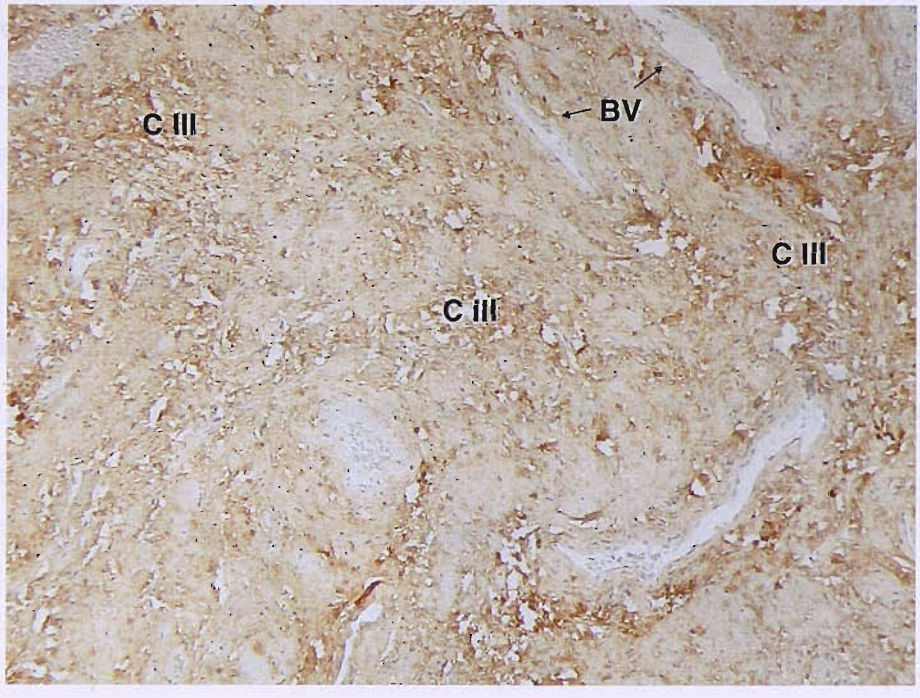


Figure 6.12 Collagen III staining for BSI group, in brown. Blood vessels are also seen (BV). Microscope magnification 10X

6.4 Analyses of collagen I and II

A Zeiss Image Analysis System was used to analyse the staining of collagen I and collagen III in the slides. The Image analysis system includes a microscope (Zeiss Axioskop 2 MOT) and a camera (Zeiss Axiocam). The computer program used was KS400.30 from Image Associates.

Three different areas of each slide were analysed. Those areas were chosen randomly to avoid any subjectivity from the researcher. The shape of the sample was drawn on a separate paper. It was covered with a grid on an acetate with numbered boxes. A computer program was used to generate random numbers. Using the first number for the rows and the second number for the columns, an area of the sample was selected. That area of connective tissue was then analysed with the Image Analysis System. The epidermis was excluded from analyses. This procedure was repeated three times for each sample so that a total of three different areas of one slide were studied.
Collagen I and III were analysed as a percentage of the captured image based on the red, green, blue colour composition of the staining.
The results of collagen I for one sample (expressed as a percentage of collagen I) was divided by the result of collagen III (expressed as a percentage of collagen III) to obtain the ratio.

$$\frac{\text{Percentage of Collagen I (\%)}}{\text{Percentage of Collagen III (\%)}} = \text{Ratio collagen I/III}$$

The ratio was then used for statistical analyses to compare the different groups.

The image analysis was performed always by me and during a period of consecutive days to maximise the consistency in the analysis and reduce subjectivity.

Reproducibility was checked by analyzing some if the slides (using the same three areas which were chosen randomly) in three different occasions. The coefficient of variation varied between 9 and 15% and it was obtained as follow:

$$CV\ \% = \frac{\text{Standard Deviation of the mean}}{\text{Mean}} \times 100$$

RESULTS

Chapter 7

Enzyme immunoassay techniques

Patients' details

Patient's details

A total of 46 patient samples were available for the different races SI study and eleven pairs of patient samples in the stretched and non-stretched prolapse study. The end number of women included in each study group varied for each assay as some samples had to be excluded from the analysis if the amount or quality of the sample extracted did not meet the strict criteria needed for the assay or were not available at the time of assay. Nevertheless when tissue available reached our criteria there were always a minimum of seven patient samples in each study group for any statistical analysis.

Therefore, patient details have been summarised for each results or chapter section.

Data regarding hormonal status, age, usage of HRT, parity and smoking habits were obtained.

A total of 11 women were recruited for the stretched and non-stretched vaginal tissue in white women with prolapse. The details have been described in each chapter according to the tissue available for each assay.

Enzyme immunoassay technique

7.1 Patient's details

7.1.1 Stress incontinence in different races study

A total of 8 women were included in the WC group, 8 in the WSI group, 9 in the BC and 8 in the BSI. All groups had a majority of premenopausal women. We found 5 premenopausal women in each of the white group and 8 in each of the black group. The only three postmenopausal women who were taking HRT during the study were in the WSI group.

There were only two nulliparous women in the white control group and 1 in the BC group. Both WSI and BSI had multiparous women only. The mean parity in the WC was 1.8, 3.2 in the WSI group, 2.3 in the BC group and 3.5 in the BSI group. There were no statistically significant differences in parity between the four groups, Analysis of Variance, ANOVA $p>0.05$.

The WC group had 3 women who were smokers and also 3 in the WSI group. The BC group had only one smoker and there were none in the BSI group. The mean age among the groups was not statistically different (mean +/- sem, WC 47+/- 5.18, WSI 49 +/- 4.7, BC 40 +/- 4.7, BSI 44 +/- 1.72; ANOVA $p>0.05$).

Group	N	Age mean (range)	Premenopausal	Postmenopausal	HRT	Parity mean (range)
WC	8	47 (43-72)	5	3	0	1.8 (0-4)
WSI	8	49 (34-68)	5	3	3	3.2 (2-5)
BC	9	40 (23-62)	8	1	0	2.3 (0-6)
BSI	8	44 (35-46)	8	0	0	3.5 (2-5)

Table 7.1 Summary of patient details for the Stress incontinence in different races study.

7.1.2 Stretched and non-stretched vaginal tissue in white women with prolapse

Eleven women were recruited for this study. Two samples for each woman were obtained. Their demographic data has been summarised in the following table.

Age	Menopause		HRT	Topical estrogens	Parity	Smokers
Mean (range)	Pre	Post			Mean (range)	
60(45 – 79)	2	9	3	0	3 (1 – 6)	2

Table 7.2 Demographic data of women in the stretched and non-stretched study

All women had vaginal deliveries. Two of them had a forceps delivery with the first baby and then had a subsequent normal vaginal delivery.

From all the women, three had jobs outside home, as a care assistant, estate agent and house keeper. Five women were retired, and three were housewives.

7.2 Total MMP-2 (This assay was only carried out for prolapse study)

7.2.1 Stretched and non-stretched vaginal tissue in white women with prolapse

Total MMP-2 was related to total protein in the samples studied (ng/mg). The MMP-2 concentration was divided by the protein concentration as obtained in chapter 3.

There were no statistically significantly differences between total MMP-2 per unit of protein between stretched compare the non-stretched samples (mean +/- sem, 10.8 +/- 1.21 v 9.6 +/- 0.88 respectively; $p>0.05$). Paired t-test was used for this statistical analysis.

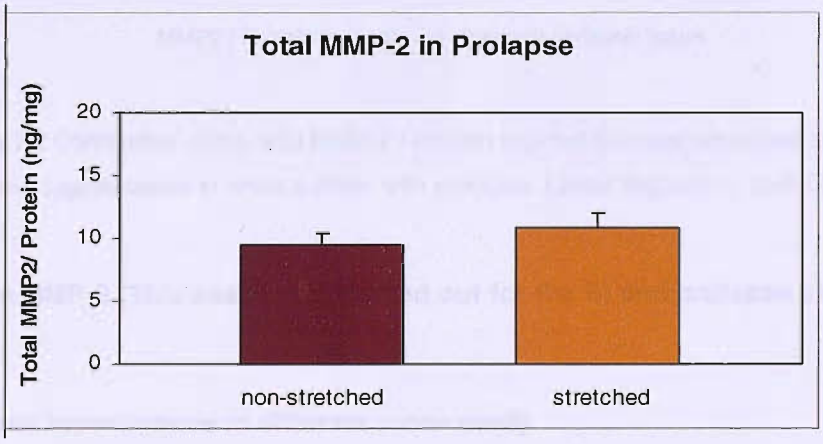


Figure 7.1 Total MMP-2 per unit of protein for the non-stretched and the stretched group (mean +/- sem, 9.6 +/- 0.88 v 10.8 +/- 1.21 respectively; Paired t-test; $p>0.05$)

Nevertheless, when linear regression was used, to compare non-stretched result with stretched a statistically significant correlation ($p=0.026$) of total MMP2 per unit of

protein between the non-stretched and the stretched samples was found. This indicated that when total MMP-2 is high, it is elevated in both stretched and non-stretched vaginal tissue, thus level of 'total' MMP-2 is inherent in the whole of the vaginal prolapse.

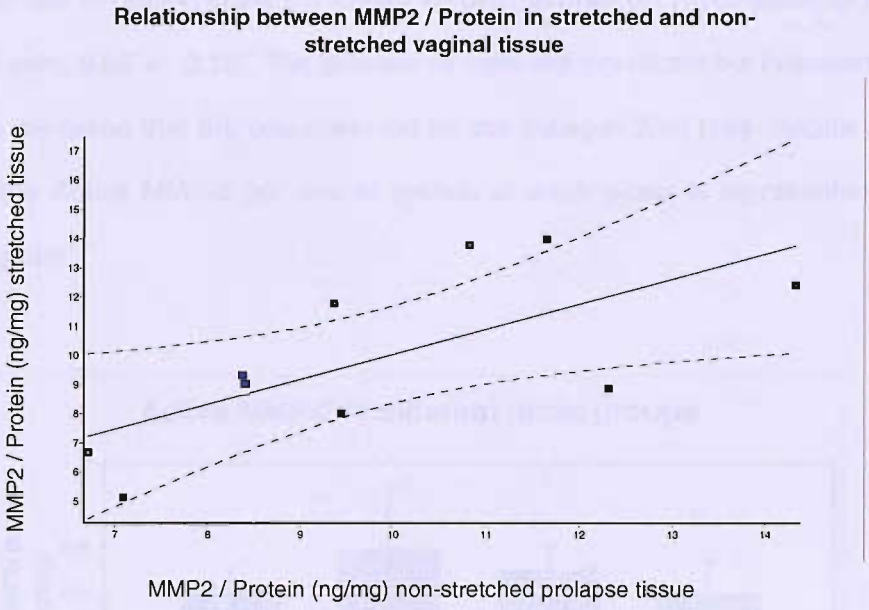


Figure 7.2 Correlation of the ratio MMP-2 / Protein (ng/mg) between stretched and non-stretched vaginal tissue in white women with prolapse. Linear regression ($p<0.05$) n=10.

7.3 Active MMP-2 (This assay was carried out for the SI and prolapse studies)

7.3.1 Stress incontinence in different races study

Active MMP-2 was related to the total protein (ng/mg) extracted from each sample weighing between 28 and 32 mg.

There were no significant differences for active MMP-2 observed among groups (ANOVA) because of the variability in each group. Mean active MMP-2 was higher in the WSI (mean +/- sem, 0.80 +/- 0.29) compared to the WC group (mean +/- sem, 0.62 +/- 0.12) and for the control groups, there was a slight increase in the level of active MMP-2 per unit of protein in the BC (mean +/- sem, 0.71+/- 0.11) compared to the WC (mean +/- sem, 0.62 +/- 0.12). The differences were not significant but interestingly the trend was the same that the one observed for the collagen XVII (see chapter 8). The mean of the Active MMP-2 per unit of protein of each group is represented in the following graph.

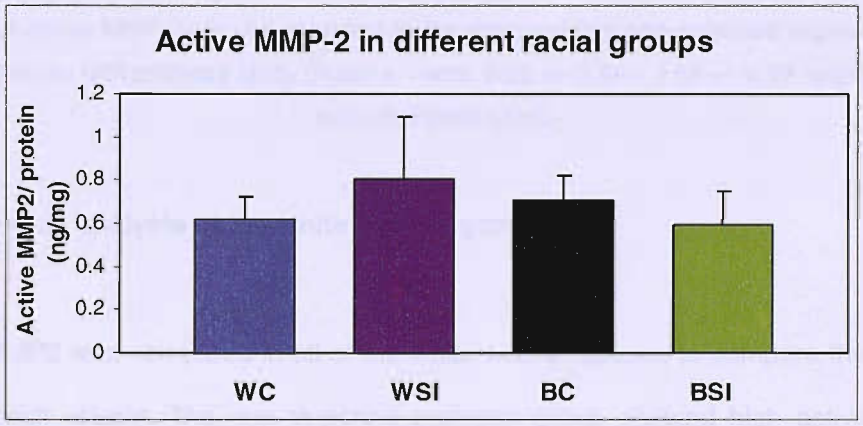


Figure 7.3 Mean and sem of active MMP-2 /Prot (ng/mg) for the four racial groups (WC, WSI, BC and BC, mean +/- sem 0.62 +/- 0.12, 0.80 +/- 0.29, 0.70 +/- 0.11, 0.59 +/- 0.16, respectively; ANOVA p>0.05)

7.3.2 Stretched and non-stretched vaginal tissue in white women with prolapse

Active MMP-2 was again related to total protein extracted in the samples. Active MMP2 was statistically significantly increased in the non stretched group compared to the

stretched group (mean +/- sem, 3.55 +/- 0.54 v 1.69 +/- 0.28 respectively; p<0.02, Paired t-test).

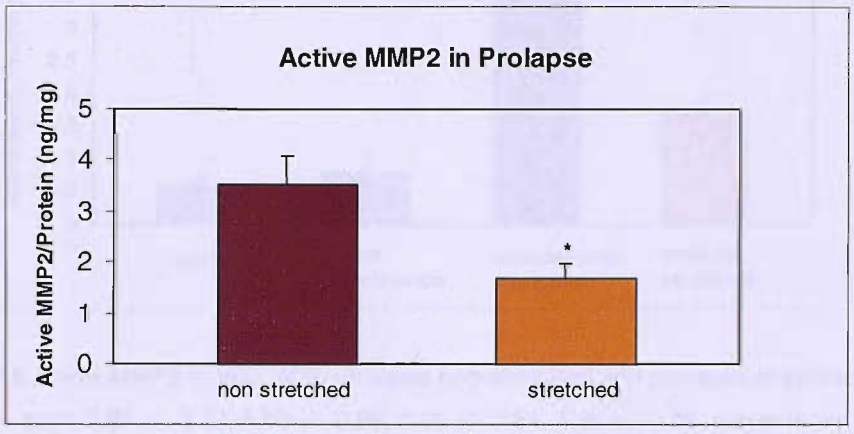


Figure 7.4 Active MMP-2 per unit of protein in the stretched and non-stretched vaginal tissue in white women with prolapse study (mean +/- sem, 3.55 +/- 0.54 v 1.69 +/- 0.28 respectively; p<0.02, Paired t-test)

7.3.3. Overall analysis of the white women group

Active MMP2 was compared in all of the white women groups to compare the severity of the insult effects. The non-stretched prolapse group showed high active MMP-2 especially compared to the white control (mean +/- sem, 3.55 +/- 0.54 v 0.62 +/- 0.12, p< 0.01)

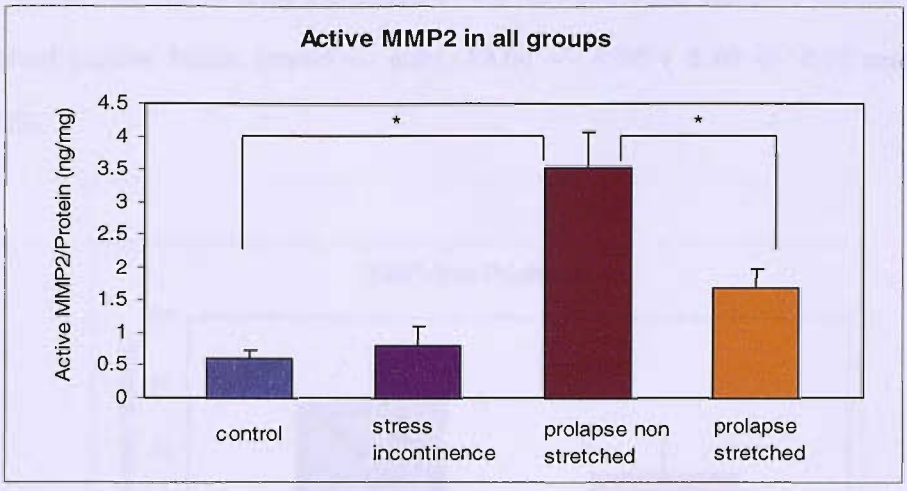


Figure 7.5 Active MMP2 in WC, WSI, prolapse non-stretched and prolapse stretched groups (mean +/- sem, 0.62 +/- 0.12, 0.80 +/- 0.29, 3.55 +/- 0.54, 1.69 +/- 0.28, respectively, ANOVA $p<0.02$)

Tukey-Kramer Multiple Comparisons Test compared each group individually and it was found that active MMP-2 in the non stretched group was significantly increased when compared to the WC groups ($p<0.01$), the WSI ($p<0.01$) and prolapse stretched group ($p<0.01$). No significant differences were found when the prolapse stretched group was compared to the WC and WSI group or when WC was compared to WSI ($p>0.05$) group (Tukey-Kramer Multiple Comparisons Test)

7.4 TIMP-2 (this assay was only carried out for prolapse study)

7.4.1 Stretched and non-stretched vaginal tissue in white women with prolapse

The results of TIMP-2 were expressed in relation to total protein content.

TIMP-2 was not significantly increased in the non-stretched samples compared to the stretched vaginal tissue (mean +/- sem, 13.64 +/- 4.08 v 8.40 +/- 2.72 respectively; p>0.05).

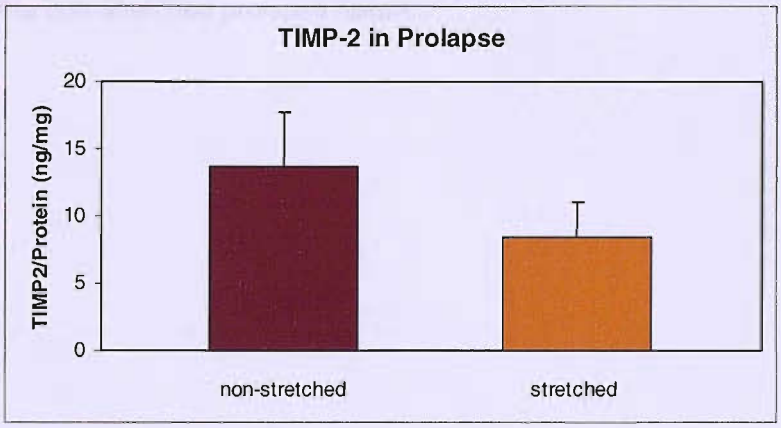


Figure 7.6 TIMP-2 per unit of protein in stretched and non-stretched vaginal skin of white women with prolapse

Summary of Chapter 7

No significant differences for the total MMP2 or TIMP2 per unit of protein were found when comparing stretched with non stretched prolapse vaginal tissue.

However, interestingly, active MMP2 was significantly increased in the non stretched prolapse group compared to the stretched group. This possibly indicates that the stretched tissue is stopping further collagen breakdown to avoid complete breakdown or a tear, or no more remodelling is possible in the stretched tissue.

Active MMP-2 was also measured in our stress incontinence and different races study but no significant differences among the groups were found. However, this did enable us to compare the white groups from the races study with the prolapse material for interest. As expected the prolapse tissue had significantly higher active MMP-2 levels especially in the non-stretched prolapse tissue.

RESULTS

Chapter 8

Molecular biology

Molecular biology

Collagen XVII message was measured by extracting initially the messenger RNA and converting it into cDNA. The cDNA was amplified by real time PCR (polymerase chain reaction).

8.1 Patients' details

8.1.1 Stress incontinence in different races study

Ten women were included in the WC group, 7 in the WSI, 10 in the BC and 8 in the BSI group. Their details are summarised in the following table (table 8.1).

Group	n	Age mean(range)	Premenopause	Postmenopause	HRT	Parity mean(range)
WC	10	47 (21-72)	8	2	1	2 (0 -4)
WSI	7	49 (34 – 63)	4	3	2	3.7 (2-6)
BC	10	40 (25 – 62)	8	1	0	2.5 (0-6)
BSI	8	46 (35 – 59)	8	0	0	3.5 (2 -5)

Table 8.1 Patient's details for collagen XVII assay in the stress incontinence in different races study

There were no statistical significantly differences in parity (mean +/- sem, WC 2 +/- 0.42, WSI 3.07+/- 0.32, BC 2.5 +/- 0.57, BSI 3.37 +/- 0.53, ANOVA p >0.05) and age

(mean +/- sem, WC 47 +/- 4.76, WSI 49+/- 4.37, BC 40 +/- 3.5, BSI 46 +/- 3.9; ANOVA p >0.05) among the groups.

There were 3 women who smoked in the WC and WSI groups, one woman smoked in the BC group while none of the women in the BSI group were smokers.

8.1.2 Stretched and non-stretched vaginal tissue in white women with prolapse

Eight matched patient samples were available for the Collagen XVII assay. Their patient's details are summarised in the following table.

Age Mean (range)	Menopause Pre	Post	HRT	Topical estrogens	Parity Mean (range)	Smokers
58(45 – 74)	2	6	2	0	3 (1 – 6)	1

Table 8.2 Demographic data for the patients included in the collagen XVII assay for the stretched and non-stretched vaginal tissue in white women with prolapse

All the women had normal vaginal deliveries apart from one who had a forceps delivery with her first child.

8.2 Collagen XVII

8.2.1 Stress incontinence in different races study

All four group (WC, WSI, BC, BSI) results are represented in the figure below. Analysis of Variance (ANOVA) was used to analyze the four groups and a p value of 0.0196 was obtained showing significant differences were present among the groups.

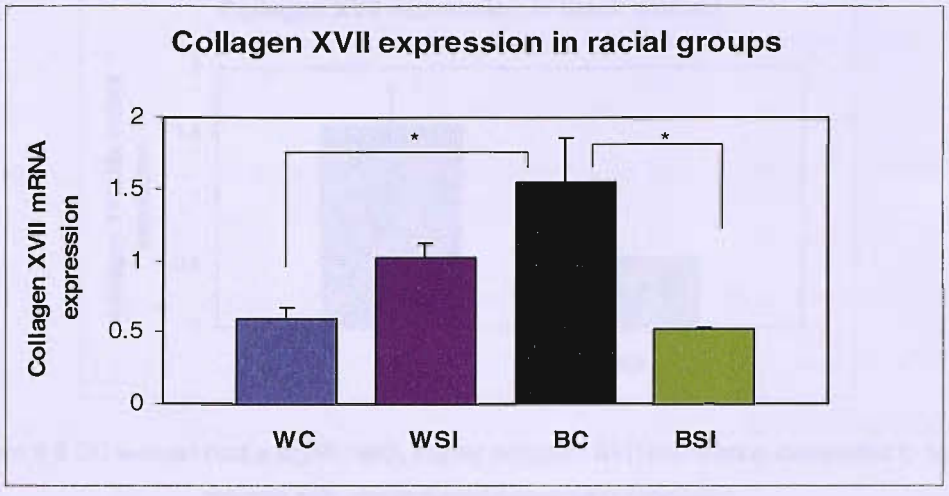


Figure 8.1 Analysis of collagen XVII mRNA expression normalized to ribosomal 18s in white and black women with and without stress incontinence (mean +/- sem; WC 0.58 +/- 0.08, WSI 1.02 +/- 0.18, BC 1.55 +/- 0.38 and BSI 0.51 +/- 0.01; ANOVA $p < 0.02$)

Tukey-Kramer Multiple Comparisons Test found a significant difference between the control groups (WC vs BC) and the black groups (BC vs BSI). They are represented again for clarity in separate figures below.

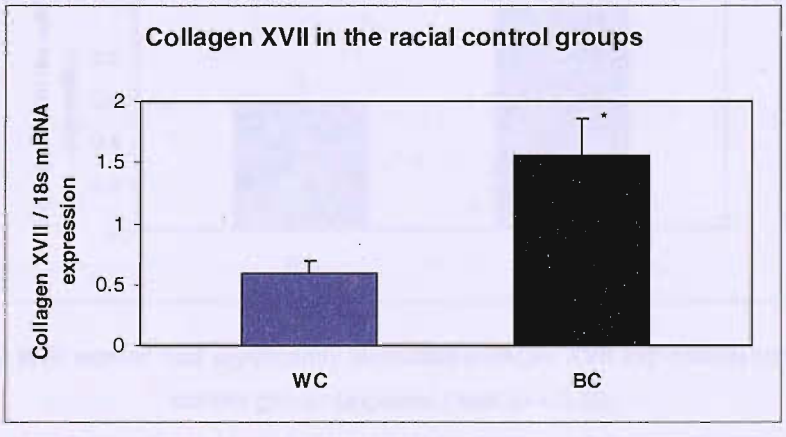


Figure 8.2 Collagen XVII expression in BC women was significantly higher than in WC women ($p<0.05$)

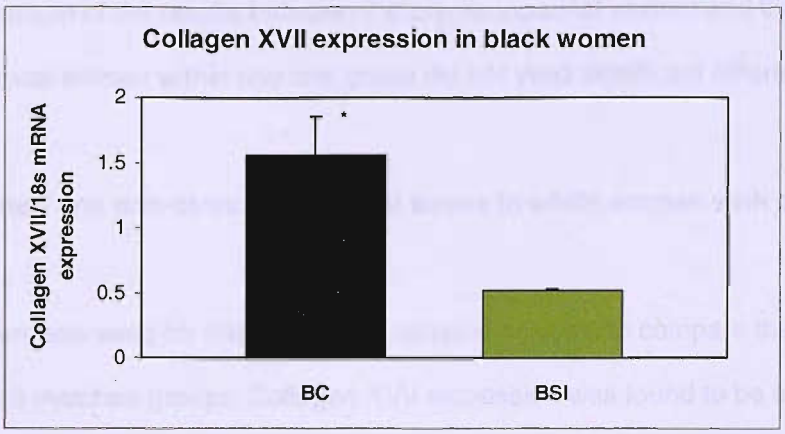


Figure 8.3 BC women had a significantly higher collagen XVII expression compared to black women with stress incontinence using ($p<0.05$)

Despite WC and WSI were found not to be significantly different when Tukey-Kramer Multiple Comparisons Test was used, for interest, if the groups were compared separately collagen XVII was significantly increased in the WSI group compared to its control group (1.02 +/- 0.18, n=7 v 0.58 +/- 0.08, n=10; p=0.02, Unpaired t-test).

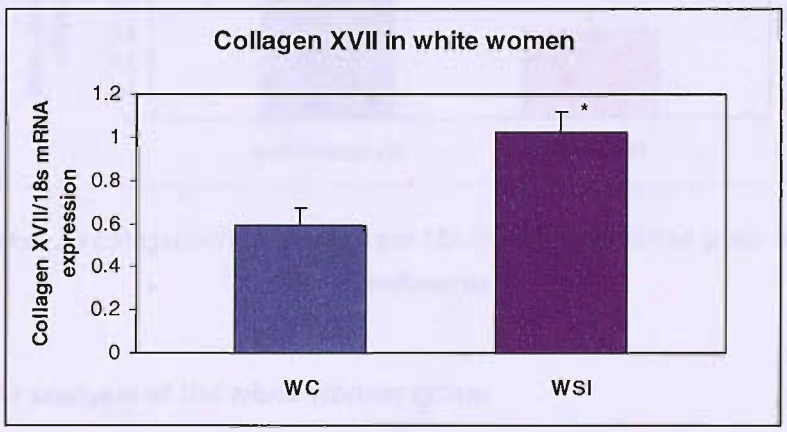


Figure 8.4 WSI women had significantly increased collagen XVII expression compared to control group. Unpaired t-test ($p < 0.02$)

Although it is difficult to draw definite conclusions of differences between pre and post menopausal women because of the small number of post menopausal available in the

study, comparison of the results between the premenopausal women and the postmenopausal women within any one group did not yield significant differences.

8.2.2 Stretched and non-stretched vaginal tissue in white women with prolapse

A paired t- test was used for this particular statistical analysis to compare the difference between the 2 matched groups. Collagen XVII expression was found to be significantly increased in the non stretched group compared to the stretched samples respectively (mean +/- sem, 1.36 +/- 0.23, n=9 v 0.59 +/- 0.11, n=9; $p< 0.02$).

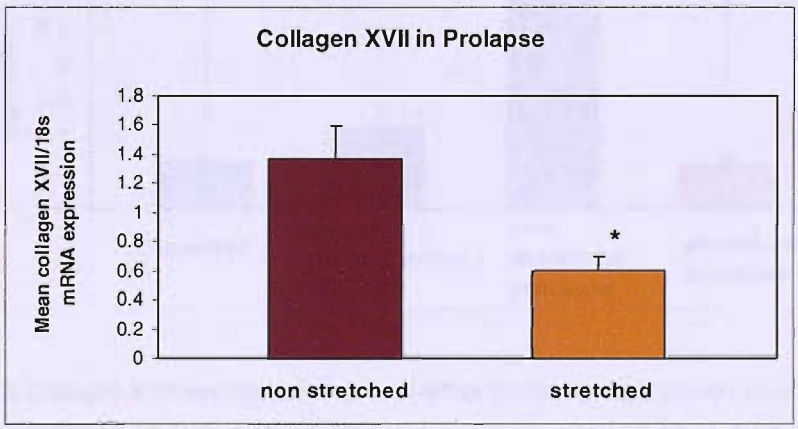


Figure 8.5 Mean of collagen XVII expression per 18s in the non-stretched group compared to the stretched one

8.2.3 Overall analysis of the white women group

For interest all white women groups (WC, WSI and prolapse samples) were compared to assess if collagen XVII expression increased with the severity of an insult. As results found in white women show a different composition in connective tissue between black

and white and a different response to tissue insult, only white groups were analyzed together to understand the response of connective tissue to different conditions affecting the pelvic floor.

Interestingly, collagen XVII expression in 'non-stretched' prolapse vaginal tissue was significantly higher than the white control group (2.95 +/- 0.83 v 0.65 +/- 0.1, $p< 0.05$), see details below.

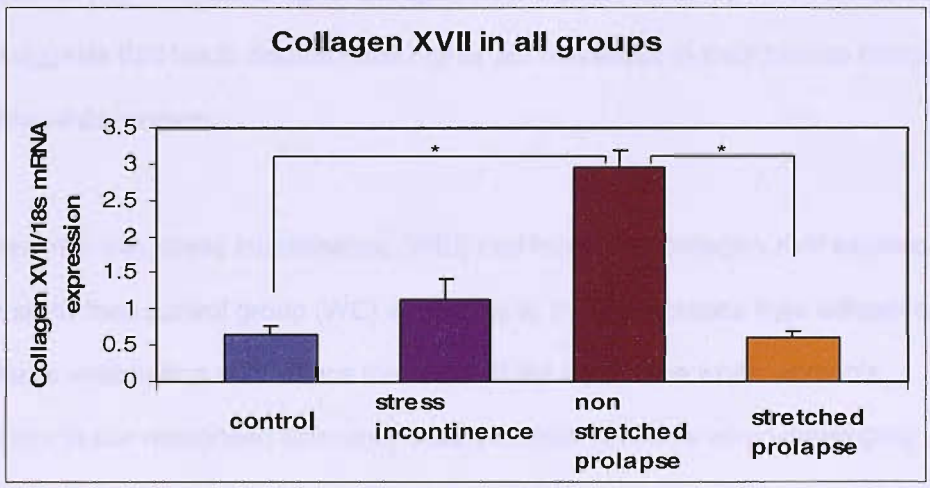


Figure 8.6 Collagen XVII expression results in white women groups (mean +/- sem; control 0.65+/-0.10, WSI 1.13+/-0.26, prolapse non stretched 2.95 +/- 0.83, prolapse stretched 0.59+/-0.11)

Tukey-Kramer Multiple Comparisons Test analysis showed a significantly difference with a p value lower than 0.05 between the control and the non stretched group and the non stretched compared to the stretched one.

Interestingly, the same pattern was observed for active MMP2 (see chapter 7) indicating that the tissue with the highest levels of collagen metabolism markers was

found in the non-stretched prolapse tissue and levels were significantly reduced when the tissue was stretched. Tissue from women suffering from SI had increased collagen metabolism markers compared to the control group, although there is not always a statistical significance.

Summary of Chapter 8

Black control women have a higher collagen XVII expression compared to white control which suggests that black women have higher adhesiveness in their tissues compared to healthy white women.

White women with stress incontinence (WSI) had increased collagen XVII expression compared to their control group (WC) appearing to try and increase their adhesiveness to increase remodelling and reduce the effect of the insult. The white woman's connective tissue responded differently than the black woman's when developing stress incontinence as increasing adhesiveness further appears not to be an option for black women and a drop in collagen XVII is seen when SI occurs in black women. Perhaps a white woman's skin is more adaptable but a black woman's is more resistant to trauma initially.

In women with prolapse, collagen XVII expression is higher in the non-stretched group compared to the stretched prolapse group suggesting remodelling can take place in skin exposed to a trauma but can eventually become so damaged that no upregulation of adhesiveness can occur. The latter may in fact reduce further stress on the tissue.

When comparing the non stretched prolapse group with the white control and then the white SI group significantly increased collagen XVII expression was observed. This supports the theory that increasing the insult increases the collagen XVII production to stop the insult. Eventually production declines as the insult becomes too severe as in the stretched prolapse tissue showing a similar pattern to that shown for active MMP-2 in the previous chapter.

RESULTS

Chapter 9

Immunohistochemistry

Immunohistochemistry

9.1 Stress incontinence in different races study

Collagen I and III were analyzed in the vaginal tissue samples from black and white women with and without stress incontinence. This was to look for any differences in extracellular matrix composition between the two races in vaginal skin. As mentioned in the introduction (chapter 1), collagen I is a fibrillar collagen that gives strength to the tissue while collagen III provides a more elastic component.

The quantification of collagen I and III was performed using a Zeiss Image analysis system as described in the methodology chapter (chapter 6). The percentage of collagen I and collagen III present and the ratio collagen I:III were obtained.

9.1.1 Patient's details

Thirteen women were included in the WC group, 13 in the WSI, 12 in the BC and 8 in the BSI group. Data for age, hormonal status, use of HRT, smoking habits and parity were recorded. The summary of patient data is shown in the following table.

Group	Age Mean (range)	Premenopausal	Postmenopausal	HRT	Parity Mean (range)
WC	44 (21 – 72)	10	3	1	2 (0 - 4)
WSI	50 (34 – 71)	7	6	4	3 (2 - 6)
BC	37 (23 – 54)	11	1	0	2.5 (0 - 60
BSI	44 (35 - 52)	8	0	0	3.3 (2 - 5)

Table 9.1 Summary of patient data for each racial group included in the immunohistochemistry study

There were no statistically significant differences in age among the four groups (mean +/- sem, WC 44+/- 3.9, WSI 50+/- 3.43, BC 43.8 +/- 1.72, BSI 43.8 +/- 1.72, ANOVA $p>0.05$).

Parity was not statistically significantly different between the four groups (mean +/- sem, WC 2 +/- 0.42, WSI 3 +/- 0.32, BC 2.5 +/- 0.57, BSI 3.3 +/- 0.53 ANOVA p= NS)

There were 7 women who were smokers in the WC group, 4 in the WSI and 1 in the BC. There were no smokers in the BSI group.

9.1.2 Collagen I

Collagen I expression in the tissues among all four groups (WC, WSI, BC and BSI) was not quite statistically significant with a p value of 0.09 (ANOVA). The means and standard error of the means are shown in the following figure.

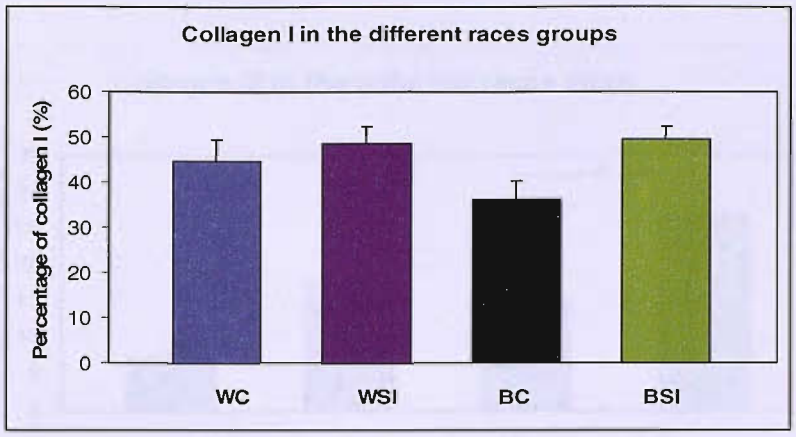


Figure 9.1 Collagen I staining in the four groups of the different races study. (mean +/- sem: WC, 44.81 +/- 4.69, WSI 48.53 +/- 3.71, BC 36.32 +/- 3.94, BSI 49.50 +/- 2.84; ANOVA p> 0.05)

9.1.3 Collagen III

Collagen III expression by immunohistochemistry was however statistically different among the groups (WC, WSI, BC and BSI with a p value of 0.0016, ANOVA). Tukey-Kramer Multiple Comparisons Test showed that BSI had a significantly increased collagen III percentage compared to the BC group (p< 0.001). Values are represented in the following table:

Collagen III	MEAN	SEM	N
WC	7.14	1.39	13
WSI	17.75	2.97	13
BC	15.04	3.44	16
BSI	26.82	3.22	8

Table 9.2 Summary of the percentage of staining for Collagen III in the four groups of the different races study expressed as a mean and standard error of the mean (SEM)

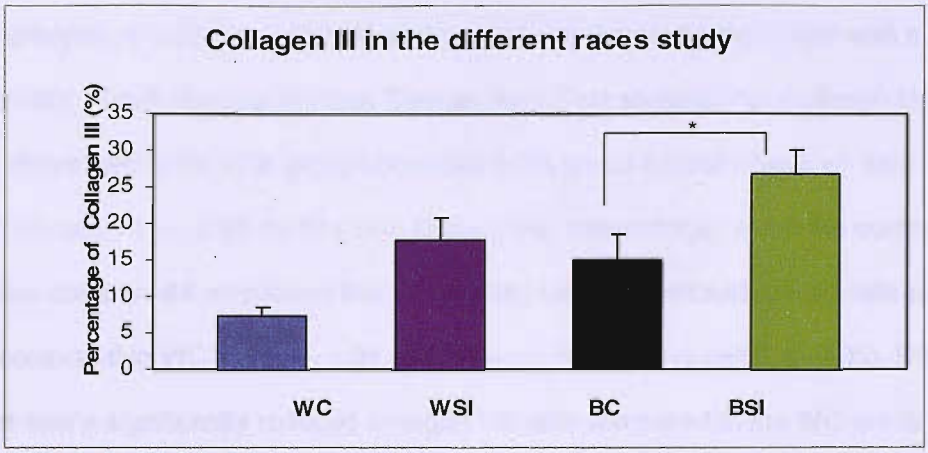


Figure 9.2 Collagen III immunohistochemical expression in the four groups of the study; ($p < 0.002$, ANOVA)

9.1.4 Collagen I:III ratio

More valuable in indicating the nature of the tissue characteristics is the ratio between both Collagens I and III. The ratio between the relatively rigid and the more elastic fibres will reveal the global properties of the tissue.

The collage I:III ratio results per each group is summarised in the following table.

Collagen I/III	MEAN	SEM	N
WC	7.91	1.19	13
WSI	3.71	0.53	13
BC	4.75	1.01	12
BSI	2.12	0.33	8

Table 9.3 Summary of the results for collagen I :III for the four groups included in the different races study expressed in mean and standard error of the mean

One way analysis of variance (ANOVA) was considered extremely significant with a p value of 0.0005. Tukey-Kramer Multiple Comparisons Test showed that Collagen I:III ratio was decreased in the WSI group compared to its group control (mean +/- sem 7.97 +/- 1.19 vs 3.71 +/- 0.53, n=13 vs n= 13 $p< 0.05$). Interestingly, when the control groups were compared it was found that BC women had a significantly lower collagen I:III ratio compared to WC (4.75 +/- 1.01 vs 7.97 +/- 1.19, n=16 vs n=13, $p<0.05$). BSI group had also a significantly reduced collagen I:III ratio compared to the WC group (2.12 +/- 0.33 vs 7.97 +/- 1.19 n= 8 vs n=13; $p<0.001$).

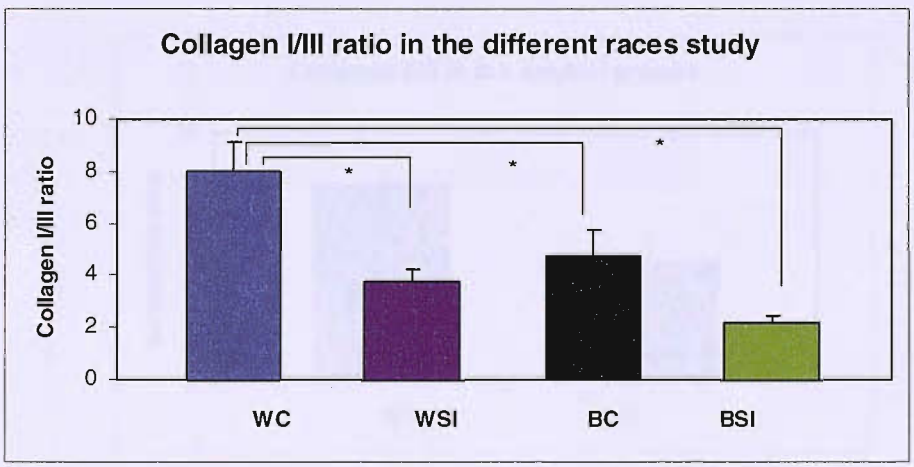


Figure 9.3 Collagen I:III ratio among the four groups included in the different races study. WC group was significantly increased when compared to any of the other group

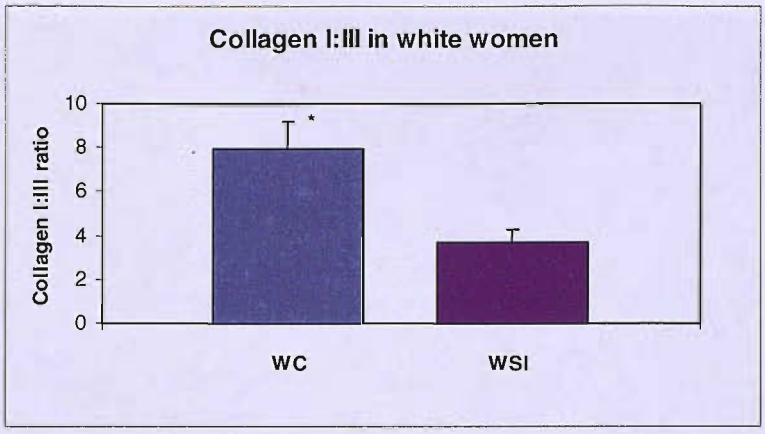


Figure 9.4 Mean of the ratio between collagen I:III in the white control group (WC) compared to the white stress incontinence (WSI) (mean +/- sem 7.9 +/- 1.19 vs 3.71 +/-0.53, n=13 vs n= 13, Tukey-Kramer $p< 0.05$)

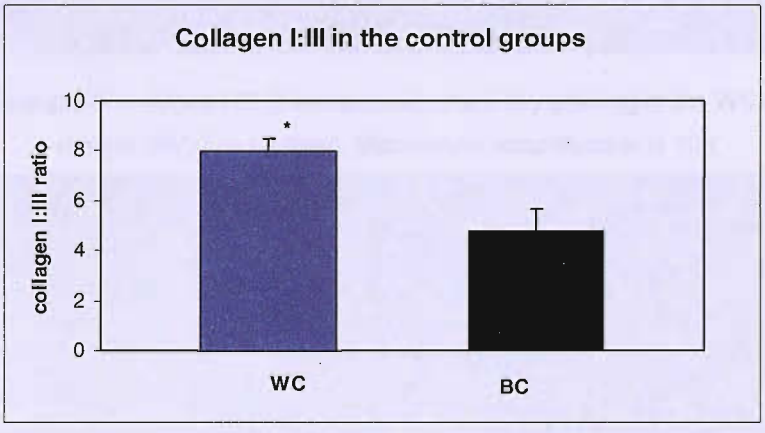


Figure 9.5 Mean of collagen I:III ratio in WC compared to BC group. (7.9 +/- 1.194 vs 4.37 +/- 0.95,n=12 vs n=13, $p<0.05$)

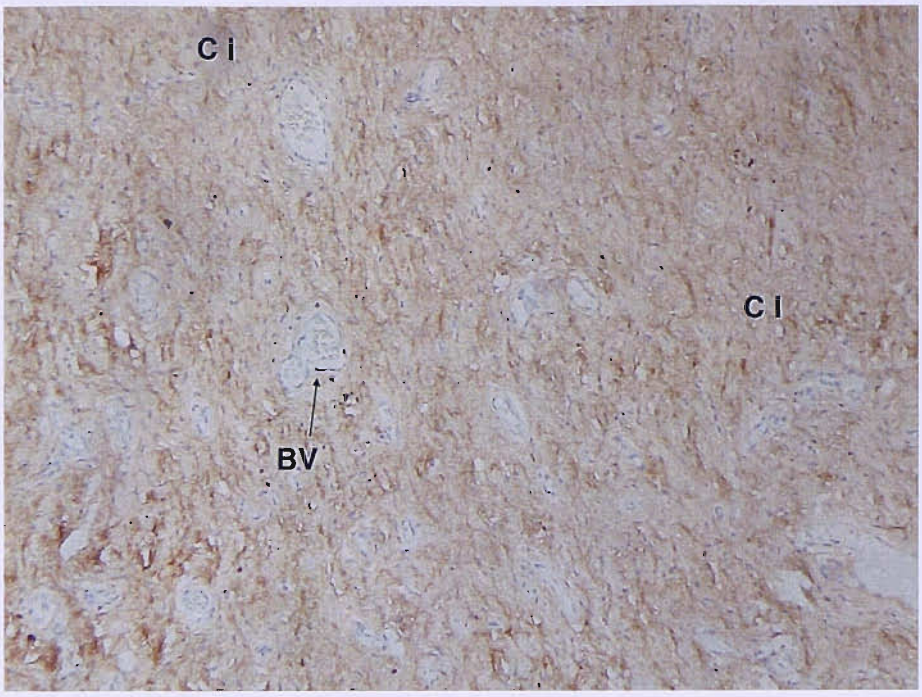


Figure 9.6 Example of collagen I (C I) immunohistochemistry staining in the WC group. Blood vessels (BV) can be seen. Microscope magnification is 10X

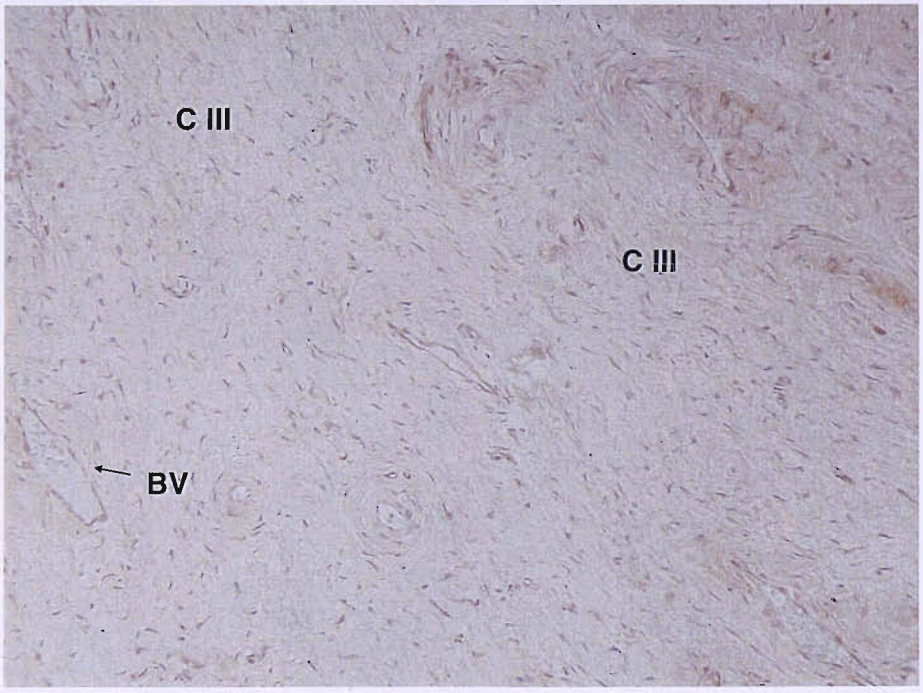


Figure 9.7 Example of collagen III (C III) immunohistochemistry staining (brown) from dermis of vaginal skin from WC group. A blood vessel (BV) is identified. Microscope magnification is 10X. Much less staining is observed for collagen III than the obtained for collagen I.

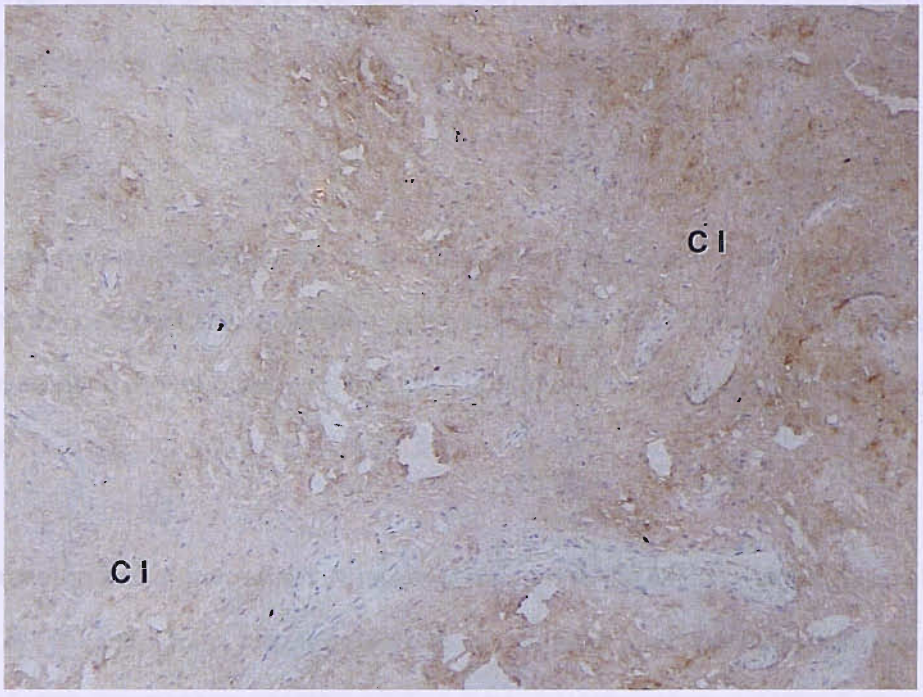


Figure 9.8 Example of collagen I (C I) immunohistochemistry staining in the BC group. Microscope magnification is 10X

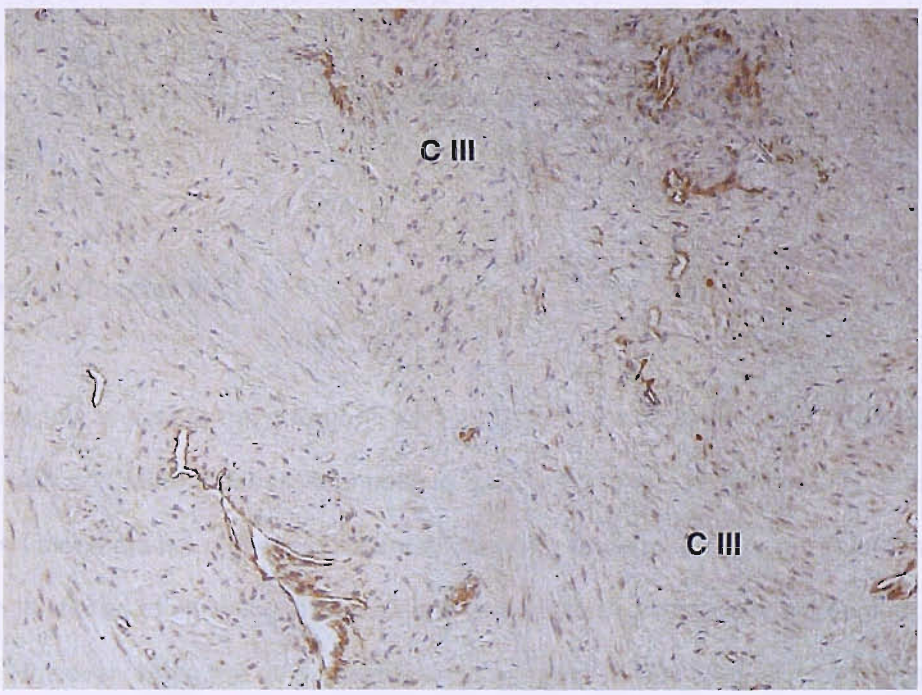


Figure 9.9 Example of collagen III immunohistochemistry staining in the BC group at a microscope magnification of 10X. A denser brown staining can be observed compared to figure 9.7 in the connective tissue of vaginal dermis.

Tissue characteristics of vaginal skin will be determined by the ratio between both types of collagen as collagen I will provide strength and collagen III will provide elasticity.

To evaluate the possible influence of hormonal status, analysis was performed excluding the postmenopausal women. Analysis of Variance still showed a significant difference among the four groups with a $p < 0.002$ (mean +/- sem, WC 8.35 +/- 1.53, WSI 3.97 +/- 0.61, BC 3.03 +/- 0.69, WSI 2.12 +/- 0.33).

White premenopausal women with SI were compared to the white postmenopausal women of the same group. No statistical differences were found between both groups (mean +/- sem, premenopausal WSI 3.97 +/- 0.61 vs postmenopausal WSI 3.40 +/- 0.97, Unpaired t-test, $p > 0.05$).

This result seems to demonstrate that hormonal status did not influence the results of this study.

Summary of Chapter 9

It was possible to assess the collagen I and collagen III status in our black and white women, with and without stress incontinence, study and reveal very interesting results. Collagen I alone did not reveal significant differences among the 4 groups studied while collagen III started to show significant differences appearing especially in the black women. However, when the ratio of collagen I : collagen III was studied, which gives a better overall measure of elasticity changes in the tissue, significant differences were

shown with an increase in elasticity for SI compared to control and very interestingly for an increase in elasticity in black control women compared to white control women. This could help to explain why the incidence of stress incontinence and prolapse is lower in black women compared to the white population.

DISCUSSION

Chapter 10

Discussion

Discussion

10.1 Stress incontinence in different races study

This is the first study to demonstrate biomolecular differences in structural support proteins for vaginal tissue between black and white women. Knowledge of these differences could be particularly important for developing future preventative or remedial methods for alleviating stress incontinence and vaginal prolapse.

As presented earlier, several studies published in the literature in the last decade have shown that Caucasian women have an increased prevalence of stress incontinence compared to black women [9 106 107]. These epidemiological studies have suggested inherent anatomical or physiological differences in the pelvic floor. The present study showed that black women have a lower collagen I:III ratio compared with white women suggesting that they have a more elastic vaginal skin as collagen I is more rigid while collagen III more elastic. A further molecular analysis of another collagen carried out in the present study also indicated that black women are producing more of the message (mRNA) for the production of an adhesive collagen, collagen XVII. No differences in active MMP2 were shown in black women compared with white, indicating that any differences between black and white women in vaginal tissue in regard to extracellular matrix characteristics were not due to a greater capacity of black women skin to remodel.

Black women have a higher bone mass density, reduced bone turnover and different calcium metabolism that protect them from osteoporotic bone fractures [108]..A higher abdominal and total subcutaneous adiposity but not visceral adiposity is also found in black women compared to white. These differences in body composition detected between black and white women support the present finding of differences in connective tissue in the pelvic floor.

Furthermore, an interesting study using MRI studied pelvic floor muscles in a group of twelve asymptomatic African American and white American women. Women were controlled for age and body mass index but not for physical activity, which could have had an impact in muscle bulk. However, the levator ani volume and a closer attachment of the puborectalis muscle were found to be significantly greater in the African American women than in the white group. This would contribute to differences in bladder neck position, urethral angle and pubic arch angle. Some structural changes regarding the urethral length and urethral closure pressure have been noted in a previous studies, as mentioned in the introduction [49]. Therefore the improved characteristics of the extracellular matrix would probably go hand in hand with any improved muscular characteristics of black women. All of the above factors would help in giving black women a greater protection from SI. Yet an increased muscle volume would not necessarily be protective for pelvic injury in childbirth and black women still develop less stress incontinence regardless of parity. Therefore, the improvements in extracellular matrix characteristics of black women over white may be more important than the muscle mass component although improving bulk could possibly improve a resistance to stress incontinence. This is presently being investigated with stem cell research.

To take the finding that improvements in elasticity is a factor in a black woman's resistance to pelvic problems, our research group subsequently studied elastin expression in the extracellular matrix of the same specimens analyzed in the present study. Our research showed that a black woman's vaginal skin has a higher level of elastin compared with white. The latter analysis was conducted by another research fellow from the same team using immunohistochemistry techniques.

Elastin is a protein secreted by the fibrocytes of the connective tissue into the intercellular network where in the dermis represents 5% of its dry weight. It works in connective tissue in partnership with collagen. Elastin allows the tissues to resume their shape after stretching or contracting. It is a relative stable protein due to many cross-links. Elastic fibres have been found to have an irregular and fragmented distribution in periurethral connective tissue from women with SI compared to controls that had intact elastic fibres closely connected with the collagen fibres, smooth muscle and fibrocytes [109]. These point to the fact that a loss in elasticity of tissue may be an important factor in causing stress incontinence and that remodelling of the tissue to increase the elasticity may help to rectify some of the problems caused by a trauma.

The increased elasticity of the tissues and the higher adhesion as indicated by collagen XVII expression could constitute an advantage in the tissue of black women to cope with the insults and strains that the pelvic floor is permanently subjected to. These characteristics would protect the tissue from damage and therefore reduce the amount of remodelling the tissue would be subjected to or perhaps even could be subjected to, as a black woman's skin does appear to not remodel as well when eventually reaching

pelvic damage (see collagen XVII results). This latter observation of a black woman's skin appears to mimic the same as for bone of black women as mentioned above.

Interestingly, black women tend to have lower perineal lacerations during vaginal birth than white women as shown in several studies [17;110], once again showing more resilience. More elastic tissues could reduce the amount of damage during childbirth by allowing the tissue to expand and thereafter come back to its previous state without breaking down.

However, black women tend to have higher keloid scars and stria than white population, showing perhaps in this case less remodelling once damage has resulted. The former are both related to elastic properties of the tissues. Keloid scars form due to a lack of replacement of collagen III for I, with an increase of activation of the gene for collagen III than collagen I [17].

The results found in the present study for white women with and without SI for collagen I:III ratio changes are consistent with others as previously shown in the literature. White women with SI have been found to have a lower collagen I :III ratio compared to white controls. Initially, such results might appear that increased elasticity in the tissues was responsible for the presence of SI. However, the findings of the present study comparing the black and the white women indicates that an increase in tissue elasticity is an advantage to resisting trauma. This therefore signifies that increased elasticity is not the cause but probably a reaction of tissue to remodel and attempt to develop more advantageous characteristics which will increase the capability of the tissue to cope with external influences and avoid damage. Women would decrease the collagen I:III

ratio in an attempt to increase the elasticity of their tissues Black women, as they have a more elastic tissue to begin with, are able to cope better, reducing tissue damage when receiving external insults, which would explain the reduce incidence in stress incontinence and prolapse.

In the present study the reduction in the collagen I:III ratio in white SI women was apparently achieved by an increase in collagen III expression in the SI group while previous studies have indicated breakdown of collagen in particular collagen I resulted in an improved ratio and elasticity. Total collagen was not measured in the present study and so a comment regarding total amounts cannot be made for the tissue studied for this thesis; however, it is known that when tissue is under stress it tries to remodel to remove that stress but this does not just involve the catabolism of the tissue (as signified by elevated active MMP2 in prolapse tissue) but also the synthesis of new collagen and matrix as seen by Jackson and colleagues when studying vaginal prolapse. Therefore, it is highly likely that vaginal tissue under stress is undergoing cycles of breakdown and new synthesis. The immunohistochemistry results were unexpected for collagen III but supported for the white women by the black and it is possible that the immunohistochemistry techniques (that were reproducible) used in present studies detected newly formed collagen III more readily. Equally, previous techniques may have detected old collagen more readily. Without studying this more fully, definite conclusions cannot be drawn, however, it is very interesting that all studies agree in that vaginal tissue appears to try and increase its elastic properties under stress and improve its resistance to tear.

Collagen XVII expression is also increased in white women with SI compared to their respective control group. Again, the tissue attempts to improve its qualities to cope with strain. This is supported by the additional finding that non-stretched prolapse tissue up-regulates collagen XVII in order to adapt, while the stretched prolapse tissue has gone past its chance of remodelling and the expression has plummeted.

More difficult to explain are the findings in the black SI group which had significantly lower collagen XVII expression compared to the control group. An explanation could be that because the initial connective tissue properties prior to SI development are different, much higher collagen XVII expression originally, that its capacity to adapt is limited.

White women skin could be more vulnerable to an insult but be more capable to adapt. As mentioned earlier, studies on mechanical variables in black and white women have shown that the higher bone mass of black women is associated with lower bone remodelling in the presence of skeletal resistance to PTH [111;112], therefore a similar situation could be occurring with black vaginal skin in that it has higher elastic and adhesive properties than that of white women but cannot respond and remodel as well under mechanical stress. In addition, up-regulation of extracellular components as seen in a white women's skin under stress is supported by evidence that mechanical stress can up-regulate the extracellular component of connective tissue in skin fibroblasts [113].

Collagen XVII is an important transmembrane protein involved in maintaining the linkage between the intracellular and the extracellular structural elements involved in

epidermal adhesion. Alterations in collagen XVII diminish epidermal adhesion and consequently skin blistering formation and subepidermal tissue separation. Chen et al linked collagen XVII and stress incontinence in a study using microarray assay which compared gene expression in vaginal skin of 5 women with stress incontinence compared to an age matched continent group [114]. The analysis suggested an alteration in collagen XVII in women with stress incontinence. We used molecular biology, a more sensitive technique to show that this type of collagen was synthesized in vaginal skin. We found it was significantly elevated in black vaginal skin from controls compared to white healthy women and in white women with SI compared to their controls group. Ideally collagen XVII protein would have been quantified in the tissues for a complete understanding of its metabolism by matching the protein with its cell gene expression. However, collagen XVII is shed into the extracellular matrix where it has regulatory roles which makes quantification extremely difficult.

Vaginal skin is subjected to hormonal influences. Our study included premenopausal and postmenopausal women as we were very limited by the difficulties in finding enough samples for the black stress incontinence group. Being selective for the menopausal status would have added difficulties to recruitment. There was a preponderance of premenopausal women in our study but an attempt was made to evaluate if any differences did exist and comparing the white SI premenopausal subgroup with the white SI postmenopausal subgroup no significant differences in collagen I:III ratio and collagen XVII were evident. This did suggest that any changes were mainly due to strain on the tissue and not to alterations in estrogen status.

Connective tissue can be affected by many other external influences like life style, diet and smoking. No obvious differences in diet, BMI, profession and social economical status were present. There were only apparent differences of more smokers in some groups but no obvious differences were noted in their results. Smoking is associated with premature face aging and induces matrix metalloproteinases 1 activity in the skin which degrades collagen. Nevertheless in our study we did not find any significantly differences active MMP2 for the smokers.

Active MMP2 has been analyzed previously by other researchers and found to be significantly higher in women with prolapse with or without stress incontinence. Active MMP2 was analyzed in a prolapse group of white women with the white controls and SI women. Active MMP2 was elevated in the pelvic organ prolapse but not in the SI group. However, the variability of our MMP2 results found in the SI group might indicate that the MMP2 levels are fluctuating to assist in remodelling. The increased MMP2 activity seen in vaginal prolapse could be the result of the strain by pelvic pressure in the tissue causing remodelling of the tissue. Women with SI without pelvic organ prolapse have no anatomical defect on vaginal examination. Nevertheless changes in the connective tissue metabolism surrounding the urethra are going on as discussed earlier

10.2 Stretched and non-stretched vaginal tissue in white women with prolapse

Alterations in connective tissue have been suggested as a possible cause of pelvic organ prolapse tissue, weakening tissue and compromising the support to intraabdominal structures. The pelvic floor maintains the pelvic organs (bladder, uterus and bowel) in the intraabdominal cavity and is submitted to constant strain as any increase in the intraabdominal pressure is transmitted to the pelvic floor tissues. This intraabdominal pressure could be the result of bearing down movement, intra-abdominal mass or simply increase in abdominal adipose tissue. Thus tissues are submitted to constant change and breakdown and remodelling will be necessary, according to the needs of the tissue. In fact, the tissue is likely to remodel to achieve a more advantageous position to reduce stress.

The capability of the connective tissue to remodel and adapt has been studied in several other areas like bone or vascular connective tissue or gingival extracellular matrix as a few examples. In bone, mandibular forward positioning in adult rats enhances osteogenic transition which eventually results in increased bone formation [115] and in gingival tissues, in vitro intermittent stretching resulted in changes in the synthesis of the extracellular matrix molecules and different types of collagen [113]. Fewer studies are available in reference to the role of connective tissue in the development of pelvic organ prolapse or stress incontinence. Some studies have shown alterations in connective tissue in pelvic organ prolapse. Soderberg et al found a decreased collagen concentration in young women suffering from vaginal prolapse [116]. Jackson et al found a significant increase in active MMP2 in vaginal skin of women suffering from vaginal organ prolapse compared to controls [73]. These changes have

been interpreted as the reason for prolapse. Nevertheless, the increase in collagen metabolism could well represent the result of persistent external strain in the tissues and therefore the consequence of the prolapse and not the cause. Persistent insult in the tissue could alter the collagen metabolism and therefore weaken the tissue allowing pelvic organ prolapse to develop.

Another part of the present research studied stretched and non-stretched vaginal tissue to help find indications of whether differences in collagen make-up were a cause or effect of pelvic organ prolapse. Two samples from different areas of vaginal skin were taken from the same woman. One sample represented the maximum strain of the tissue (stretched) and the other healthier tissue supporting less strain (non-stretched). They represent different stages in the development and worsening of pelvic organ prolapse as tissue is submitted to increased strain. Active MMP2, responsible for collagen breakdown was found to be significantly lower in the most stretched area compared to the vaginal tissue under less strain, the non-stretched, but close to the prolapse. This suggests that close to a strain or an initial stage of a prolapse, the tissue is at its most active and increased remodelling and collagen metabolism is being carried out to reduce the strain. When the insult persists or increases the tissue then slows down the breakdown to protect the tissue from total breakdown and tearing and so active MMP2 falls.

Total MMP2 and TIMP2 were not significantly different in the stretched group compared to the matched non-stretched one. If the total MMP2 was high in the stretched tissue it was high in its matched non-stretched. The high total MMP2 appears to be inherent in the whole of the vaginal tissue but active MMP2 was more related to

the amount of remodelling it should do as a result of the insult to the tissue. There were no significant differences in TIMP-2 in the non-stretched group compared with the stretched although, the mean was higher in the non-stretched indicating remodelling was probably going on.

There was a significant increase in MMP2 (active), as well as collagen XVII, in the prolapse tissue compared to the white controls white SI when they were compared.

This is the first study where prolapse tissue has been analyzed and compared in two different areas affected by different degree of strain and has shown differences in the active MMP-2. The results of MMP2 changes occurring in a vaginal tissue can now be summarized and are shown below. From the literature and present results it appears that total MMP2 becomes up-regulated ready for remodelling of vaginal tissue as insults to the tissue occur. It is possible that this gets used and remodelling occurs without any severe consequence with active MMP2 up-regulated and released in the stressed area. However, eventually the build up of MMP2 and breakdown becomes so great that a prolapse occurs and the active MMP2 has to be switched off to reduce further collapse of tissue structure (figure 10.1).

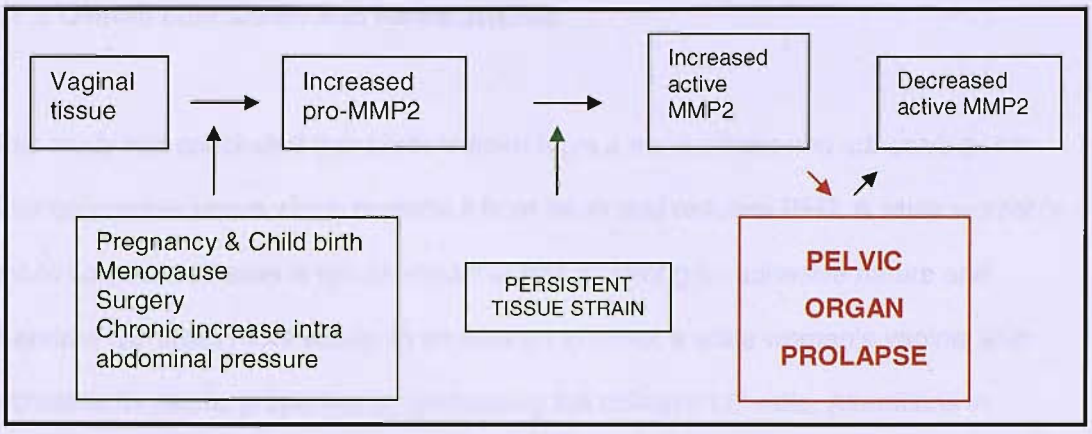


Figure 10.1 Theory of pelvic organ prolapse pathophysiology

10. 3 Overall conclusion and future studies

This study has concluded that black women have a more elastic and adhesive pelvic floor connective tissue which protects it from injury and reduces PFD. A white woman's pelvic connective tissue is not as elastic or has as strong an adhesive nature and therefore damages more easily. In an attempt to adapt a white woman's vaginal skin increases its elastic properties by decreasing the collagen I:III ratio. Alterations in connective tissue found in previous studies in women with prolapse and SI and thought to be responsible for these conditions could actually represent changes in the tissue as part of a process of adaptation after receiving repetitive insults.

Pelvic fascia is submitted to constant stimuli which have an impact in its collagen metabolism. In women with SI an increased variability in MMP2 activity levels could indicate an initial process of remodelling. As insults to the tissues persist the remodelling becomes more intense in an attempt to repair and restore the tissue. Weakened tissue loses support properties and development of prolapse occurs. As the strain to the vaginal skin persists remodelling diminishes to avoid complete breakdown of the tissues.

An inherent factor can be suggested as there was a correlation between the total MMP2 in the stretched and non-stretched group. Some women may be at increased risk of developing PFD after receiving an insult depending on their connective tissue make up. The assessment of the inherent factor is always difficult to assess. The findings in the black control group indicates that more elastic tissue can be protective for PFD. White women with increased elastic properties in their connective tissue could

be also at a lower risk of developing pelvic organ prolapse which could explain why some women develop PFD while others do not regardless of their parity or menopausal status. Tissue elasticity could be a predictor factor for PFD.

The ability to influence the remodelling of collagen could prevent deterioration of pelvic floor defects. It is extremely interesting that estrogens have the effect on the vaginal skin of maintaining its health and preventing atrophy. Yet one selective estrogen receptor modulator, raloxifene, has been shown to be related with worsening in pelvic organ prolapse in postmenopausal women [117]. In animal studies, estradiol increases the expression of collagen I and III by the fibroblast in the vaginal connective tissue[118]. Further knowledge about the role of estrogen in the regulation of collagen metabolism could open a door in the prevention of pelvic organ prolapse. However, our results signify that estrogen may need a little help and growth factors and tissue engineering to improve vaginal skin elasticity and adhesiveness may be the answer. New and exciting areas of research are now opening up and hopefully this thesis helps to point towards them. This research will ultimately lead to better ways to improve the quality of life of many women affected by the debilitating conditions of pelvic floor disorders.

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Appendix 1

Name:
ID number:
DOB:

Age:

Race:

White Caucasian	
Black African	
Asian	

BMI:

Occupation:

Socio-economic status: Education
Financial assets

Smoking: Yes / No

Diet:

Parity:

	date	mode of delivery	weight of baby	2nd stage length	PFE
1					
2					
3					
4					
5					
6					

Hormonal status: - Pre-menopause / peri-menopause /post-menopause
- LMP:
- HRT: opposed - unopposed / No HRT

Medical history:

Chronic cough	yes	no
Diabetes	yes	no
Connective tissue disorders	yes	no
Chronic constipation	yes	no
Haemorrhoids	yes	no
Varicose veins	yes	no
Urinary tract infections	yes	no
CNS disorders: MS, Parkinson....	yes	no
Spinal cord injuries	yes	no
Congenital disorders	yes	no
Lower genitourinary anomalies	yes	no
Abdominal hernia	yes	no
PID	yes	no
Degenerating fibroids	yes	no
Malignancies	yes	no
Stroke	yes	no
Ligament injury	yes	no

Previous surgery:

Hysterectomy	yes	no
Continence procedure	yes	no
Prolapse	yes	no
Irradiation	yes	no

Medication:

Diuretics	yes	no
ACE inhibitors	yes	no
Alpha blockers	yes	no
Allergy medication	yes	no

Others:

Symptoms:

Symptoms	Y / N	Duration	Severity(n° of times a day)	Coments
Stress incontinence	Y / N			
Urgency	Y / N			
Urge incontinence	Y / N			
Frequency	Y / N			
Nocturia	Y / N			
UTI	Y / N			
Prolapse symptoms	Y / N			

Others:

Urodynamics report:

- Functional bladder capacity: mls
- Frequency: Nocturia:
- Incontinent episodes per day:

- Initial voided:
- Max flow rate: ml/sec
- Average flow rate: ml/sec
- Voiding time: min/sec
- Bladder capacity: mls
- Max. bladder pressure: cm H2O
- Residual: mls
- Leak point: cm H2O
- Bladder stable during filling: Yes / No

Coments:

Examination:

Pelvic examination:

Vaginal examination:

Vaginal skin: Normal Red Sore Rash Excoriated

Vaginal mucosa: Well oestrogenised Poorly oestrogenised Atrophic

Perineum: Healthy Scarred Deficient

Prolapse	nil	Small 1st	Moderate 2nd	Large 3rd
Anterior				
Posterior				
Apical				

Abdominal striae:

Joint hypermobility: