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I, Eduardo Cort s , confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgements

Foremost, I would like to express my most sincere gratitude to my main supervisor Professor Geoffrey Goldspink for his continuous support, guidance, motivation, and immense knowledge, but above all, for making me feel a friend, which I will always appreciate from my heart. Without his help and faith in this project this work would have never been completed.

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Last, but by no means least, I would like to thank my wife Christina who has always stood by me, for her strength, love and the financial sacrifices she endured to support me during the final years of this thesis.

I would like to dedicate this work to Eva, my daughters Alexia and Maria Merce, my brother Jorge and his wife Sandra, my father (somehow) and my mother, Natividad, who sadly passed away before this work was completed.

ABSTRACT

Introduction.

Studies in skeletal muscle following damage have demonstrated splicing of the insulin-like growth factor-1 gene to IGF-1Ea that is the source of mature IGF-I which is a main anabolic factor, IGF-1Eb,the function of which is not known and IGF-1Ec, known as Mechano Growth Factor (MGF). MGF increases the pool of muscle stem cells by activating them to proliferate. The aim of the thesis was to study expression of IGF-1 variants in female reproductive organs where cellular damage may lead to pathology:

1. The levator ani (LA) muscle: significant damage occurs following vaginal delivery and is a key element in pelvic floor physiology. LA samples from pre and post menopausal women were used as controls.
2. Ovarian tissue: repeated ovulations and failure of the epithelium to repair which may be a predisposing factor for ovarian cancer.
3. Myometrium: single monoclonal cellular damage may lead to abnormal proliferation of myometrium and may be a factor in the aetiology of uterine fibroids

Methods

Samples were analysed using real-time qPCR, with specific primers for IGF-1. Immunoanalysis using polyclonal MGF antibody was performed to demonstrate tissue expression of the proteins.

Results

IGF-1 splice variants were highly expressed in LA muscle following first vaginal delivery. IGF-I Ea splice variant expression was less in the LA of post-menopausal women and MGF was not detected in this group.

MGF and IGF-1Ea were detected in malignant and non malignant ovarian tissue. MGF was found to be expressed and could be part of the repair process in this non muscle tissue.

Both IGF-I factors were present in myometrium and fibroids, however IGF-1a expression was significantly reduced under ischaemic conditions.

Conclusions

IGF-1 splice variants are apparently involved in the repair and regeneration of normal and pathological tissues and variations in their expression and interaction with other tissue repair mechanisms may contribute to the understanding of physiological and pathological tissue growth.

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Aims of the thesis

1. Identify the expression of IGF-1 splice variants in the LA muscle in women, and quantify their expression as a result of the physical stress overload following spontaneous vaginal delivery.

2. Compare expression of IGF-1 splice variants in young and elderly patients. Decreased expression of these splice variants in elderly women may explain clinical differences seen in the conservative management of incontinence.

3. Identify expression of IGF-1 splice variants in other damaged tissues in the absence of overload damage, and its presence as a repair mechanism. Tissues studied are normal and pathological ovary, myometrium and leiomyoma.

Introductory chapter

I conducted this MD thesis as a Research Registrar at the Royal Free Hospital, London (2001-2003) whilst I remained a full time on call Registrar in Obstetrics and Gynaecology. I enrolled as a part time MD student with University College London in 2003. The original project of this thesis was to describe the anatomical changes seen within the levator ani (LA) muscle during pregnancy and after labour using dynamic Magnetic Resonance Imaging (MRI), and to correlate postnatal changes with the patient s pelvic floor function. At the same time, I also worked with Dr Kavita Singh who was a lecturer in the pelvic floor unit, and had developed an expertise in the female LA muscle anatomy using newly descriptive techniques in the pelvic floor such as conventional MRI, dynamic MRI and 3D MRI.

Although most of the work conducted in the unit to that date had focused in descriptive anatomical aspects of the female pelvic anatomy in relation to age and prolapse, its value as a clinical tool in the diagnosis of compartmental prolapse had not yet been put to the test. In order to assess the latter, and the feasibility of assessment of prolapse in an MRI setting, I conducted a comparative study looking at the correlation between clinical

assessment of vaginal vault prolapse in women and MRI findings. The data used for this study was extracted from a clinical and MRI database already set up in the pelvic floor unit in women presenting with pelvic floor problems. MRI studies were part of the routine assessment in women with complex vaginal prolapse. Initial findings from the preliminary data obtained were presented in the American Urogynecolgy Association meeting in San Francisco, 2002. Following oral presentation, the abstract received very valuable feedback by experts from the audience which required the redesign of the methodology by incorporating a validating arm to the study, the intraoperative assessment. The new project developed into the paper submitted and published in the American Obstetrics & Gynecology Journal. Simultaneously with this project, I started the process of obtaining ethical approval for the originally intended work of the thesis. However it was necessary to demonstrate that the anticipated methodology would be a valid tool for the description of any findings. The following reading is a summary of the methods, results and discussion of the published manuscript.

The interim paper

Clinical examination and dynamic magnetic resonance imaging in vaginal vault prolapse[1].

For the purposes of this MRI based study 51 women ages 40 to 95 years old (mean age 64) presenting with symptomatic vaginal vault prolapse following previous hysterectomy (Vaginal 42%; Abdominal 58%) agreed to have a dynamic MRI evaluation of their pelvis preoperatively. Women attending the urogynaecology clinic underwent clinical evaluation of their prolapse, in the dorsal supine and standing position, using the Pelvic Organ Prolapse questionnaire (POP-Q) grading system as described by Bump *et al* [2], by three experienced gynaecologists. Following clinical assessment all women underwent further evaluation of their prolapse using dynamic magnetic imaging (MRI). Disagreement between clinical and MRI findings in the middle compartment was clinically re-evaluated intra-operatively. Examination was performed in theatre with the patient in semi extended lithotomy position prior to reconstructive surgery. The vaginal apex was identified, held with a tenaculum at the same time gentle traction was applied. The distance the apex descended in relation to the hymen was documented.

MRI methodology

Imaging was performed in the dorsal supine position with both legs together and supported in a semi-flexed position. All images were obtained using a 1.5 Tesla Philips Gyroscan (Phillips Medical Systems, Nederland B.V.). Static T2 TSE images were obtained at 5mm/5mm on axial and sagital sections, with a field of view of 280, matrix 230x512, TR 6086/TE150 and 4.5 minutes acquisition time per plane. Dynamic imaging in the sagital plane was performed using a fast spin echo sequence. The slices were obtained on maximal straining (increasing Valsalva manoeuvre) at 5mm/5mm, with field of view 350 and 17 seconds acquisition time per plane. In order to facilitate analysis of the films, sterile lubricating jelly (Aquagel) was inserted vaginally and rectally. This prov ided the vagina and rectum with a degree of opacity that enhances visualization. Women were imaged with a partially filled bladder and any vaginal support pessaries were removed.

Scoring system

Previous MRI studies have used different reference levels for grading prolapse, such as the pubo-sacral or pubo-coccygeal line [3] which can over-diagnose or limit diagnosis to one specific

compartment. In this study I chose the Mid Pubic Line (MPL) in the sagittal plane, as the reference level for grading prolapse on MRI. The MPL is a line drawn across the mid-sagital aspect of the pubic bone K Singh et al [4] (Fig 1) which corresponds with the level of the vaginal hymen, hence co-terminus with the clinical reference level described by Bump *et al* [2]. A simplified scoring system (Fig 2) was used to facilitate the description on MRI of the vaginal compartment involved: A = Anterior compartment; M = Middle compartment; P = Posterior compartment. The different stages of prolapse were described as ranging from 1 to 4 as referenced by distance from the MPL. Images were analysed and validated by the same two observers, using a workstation (Sun Mycrosystems, R-Ray department), with zoom facility and electronic callipers. Both observers were blinded from the clinical findings at the time of MRI evaluation. In an attempt to simplify our findings, grades 2 to 4 descent were regarded as significant prolapse and grade 0 to1 as non significant.

Statistical analysis

The Weighted Kappa test was used to compare values between both grading systems. Test for symmetry was used to assess significance of over and under-diagnosed results in each

compartment. Statistical analysis of intra-operative assessment, to confirm either clinical or MRI findings pre-operatively, was performed by using the McNemar s Chi Squared test. Statistical significance was taken as a p value of less than 0.05.

Results

Analysis of each compartment independently revealed poor correlation between clinical and MRI findings (Table 1). Only 17 out of 51 (33%) was there agreement between clinical and MR scores in the anterior and posterior compartment. In the middle compartment, agreement was seen only in 21 patients of the 51 (41%). Using the same parameters, both in the anterior and posterior compartment, 19 cases out of 51 (37%) were over diagnosed clinically and 15 cases (29%) were under diagnosed clinically, when compared to MRI findings.

When assessing the middle compartment alone adopting MRI findings as the reference diagnostic level, 27 cases (52.9%) were clinically over-diagnosed and only in 3 cases (6%), were under-diagnosed clinically. Test for symmetry showed a p = 0.002, showing a significant difference in this compartment, when

compared MRI staging and clinical assessment of prolapse. No significant differences were seen in the anterior and posterior compartment. Looking in more detail at the degree of prolapse in the middle compartment, we found that 35 of the 51 patients (69%) who had been clinically diagnosed with a moderate to severe (significant) prolapse of this compartment (M2 - M4), in 21 of these 35 cases (60%), were reclassified as normal to mild prolapse (non significant) when we looked at these patients dynamic MRI images. Interestingly, 20 of the 21 reclassified as non significant middle compartment prolapse, were found to show on MRI, different degrees of significant prolapse in the anterior and posterior compartment (A2 to A4, and P2 to P4). This also showed that in the anterior compartment, 7 (28%) of the 25 clinically diagnosed as significant prolapse were reclassified as non significant using MRI. Likewise, in the posterior compartment 9 (28%) of the 32 clinically significant posterior compartment prolapse were reclassified as non significant.

Three of the 21 patients above mentioned (14%) whose MRIs of the middle compartment reclassified them as non significant prolapse, were managed with a ring pessary. The remaining 18 patients (85%) were assessed clinically both intra-operatively and post-operatively. Clinical preoperative findings were confirmed

intraoperatively in 15 of the 18 cases (83%), and a sacrocolpopexy procedure was performed. MRI findings reclassifying middle compartment prolapse as non significant, were confirmed in 3 of the 18 cases (17%) intra-operatively and vaginal surgery for correction of anterior and posterior compartment defects was performed instead, following MRI findings. In one of the 15 cases, intra-operative findings confirmed both the clinical diagnosis in the middle compartment and agreed with the MRI findings in the anterior and posterior compartment. The patient underwent a combined abdominal and vaginal approach.

Post-operative assessment at 3 months, revealed no recurrent middle compartment prolapse in any patients. Four (26.6%) and three (20%) women out of 15 had persistent anterior (cystocele) and posterior (rectocele) compartment prolapse respectively. Although these had been detected on MRI preoperatively, intra-operative assessment was not conclusive enough to alter the initial surgical management.

Although the study revealed poor correlation between clinical and MRI findings when defining compartment descent as well as in identifying each compartment separately, it was acknowledged that

there are relevant limitations such as mobility restrictions and compartment overlapping which could condition the interpretation of future studies. These space limitations already relevant in the non pregnant population, would suggest they could become more of a restriction in a pregnant population making the case for an open MRI setting in this case a more suitable option. Intra-operative assessment should also be carefully interpreted as pelvic floor descent under general anaesthesia may not reproduce prolapse to the same extend as under physiological conditions. Although subjects were assessed pre-operatively during valsava manoeuvre, abolishment of the pelvic floor muscles support under anaesthesia, will reflect prolapse resulting from its fascial supports only, may be leading to intraoperative overdiagnose of prolapse. In the current study, intraoperative assessment was used as a validating tool between the clinical diagnosis and MR imaging of the middle compartment, confirming clinical assessment of vaginal apex descent in 83% of cases. In those patients whose vault descent was not detected intraoperatively, 3 out of 18 (17%), the prolapse was repaired in accordance with the MRI findings in the anterior and posterior compartment only. Interestingly, out of the 83% (15 over 18) who underwent an abdominal colposacropexy, patients presented post-operatively with a cystocele (26.6%) or rectocele

(20%) which although detected preoperatively using MRI, it had not been conclusively defined during either clinical or intraoperative assessment. This finding may suggest that dynamic MRI may play a more reliable role in the assessment of the anterior and posterior compartment within the context of complex vault prolapse than clinical or intraoperative assessment. Additional findings on MRI revealed peritoneum involvement in twelve cases (23%), one anterior peritoneocele, and sigmoid colon descent in two cases (4%). The clinical value of these findings remains relative as they may not necessarily generate a change in surgical approach.

The use of different imaging techniques has been explored in multiple studies since this pilot study took place. Recent expertise reflects the need for further studies in an attempt to validate the different imaging techniques for the assessment of the Levator Ani muscle, the correlation of these with pathology and the long term implications of injury in pelvic floor pathology[5].

The expedited birth of a new project

At the same time the above study was being completed, the process of applying for ethical approval on the original project followed its course and ethical approval was granted following a personal interview with the local Ethics Committee at the Royal

Free Hospital. Unfortunately when all was set up to start patient recruitment, the funding for the initial project was discontinued.

This new unexpected situation raised the need to find a new project that would allow me to continue in the position I was initially appointed for. While performing a literature search on the levator ani muscle and muscle physiology, incidentally I came across a paper by Professor G Goldspink on skeletal muscle damage following injury or over stretching, and the newly discovered IGF-1 splice variants. Fortunately, Professor G Goldspink s team was based at the Royal Free Hospital which gave me the opportunity to discuss a collaborative project involving the study of these muscle repair factors on the levator ani muscle. Based on my previous knowledge acquired using MRI on the anatomy of the levator ani muscle and pelvic floor, I designed a technique to biopsy the LA muscle in the pregnant and non pregnant population. I arranged several meetings and presentations with Professor Goldspink s team explaining the mechanisms of labour, the working hypothesis and the process of LA muscle biopsy. A new ethical approval for the study was obtained in 2002. Data collection was completed prior to my return to my full commitments in Obstetrics and Gynaecology in 2003, when I registered my MD project. Personal circumstances and taking the membership exams delayed the

submission of the thesis that follows. These circumstances were presented to University College London for consideration of their late submission fees which were waved by the Chair of the Academic committee. The study presented next is the result of the second project commenced during my research years.

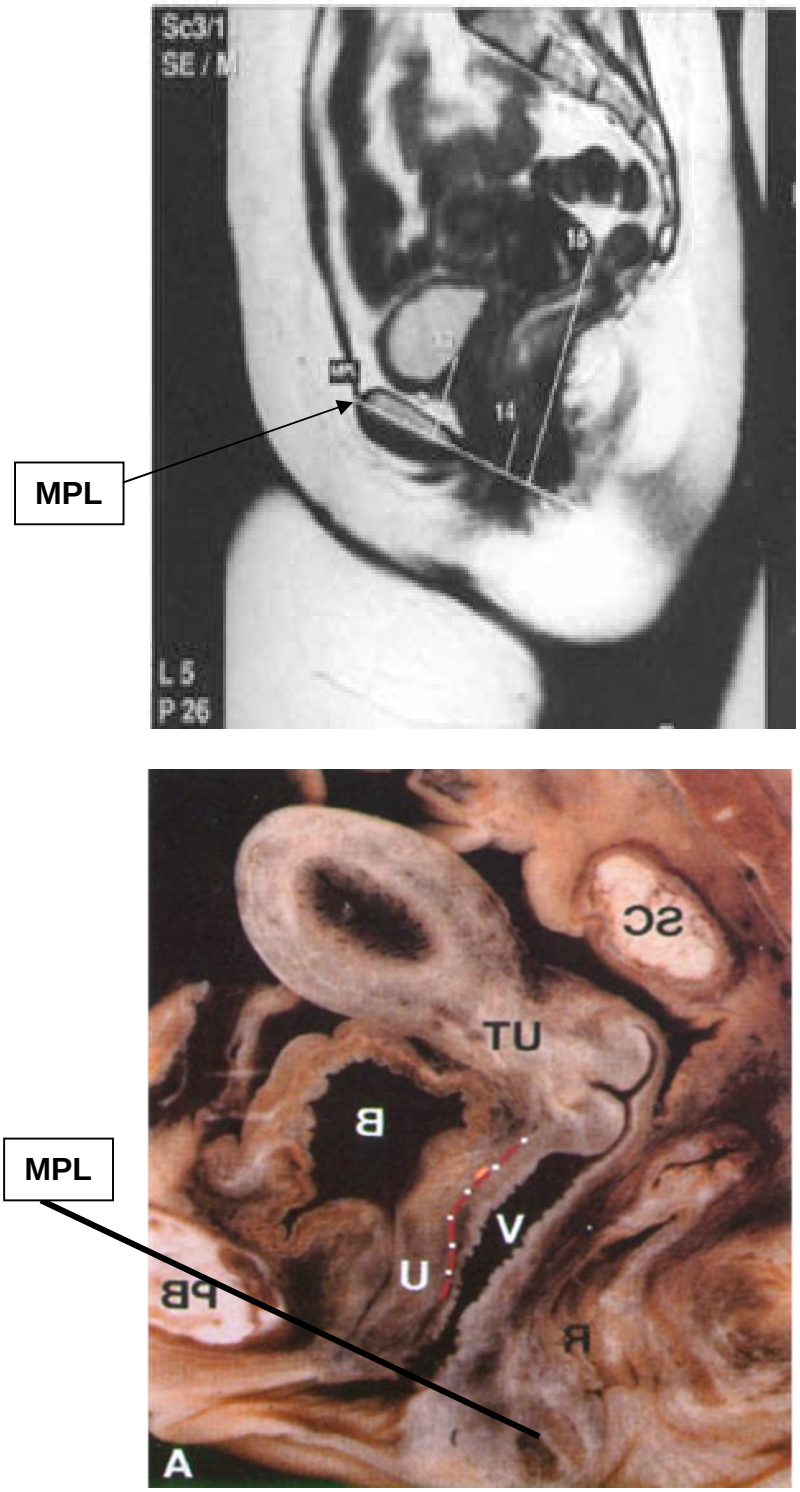

Fig 1.

Mid Pubic Line (MPL)
Picture showing a sagital view of the pelvic floor on MRI (left) and in a human cadaver (right). The MPL is used in this study as the reference line as it passes along the hymen level in the vagina, facilitating grading prolapse above and below it. *B: Bladder; UT: Uterus; V:Vagina; U: Urethra; PB: Pubic Bone; R:Rectum(1)*

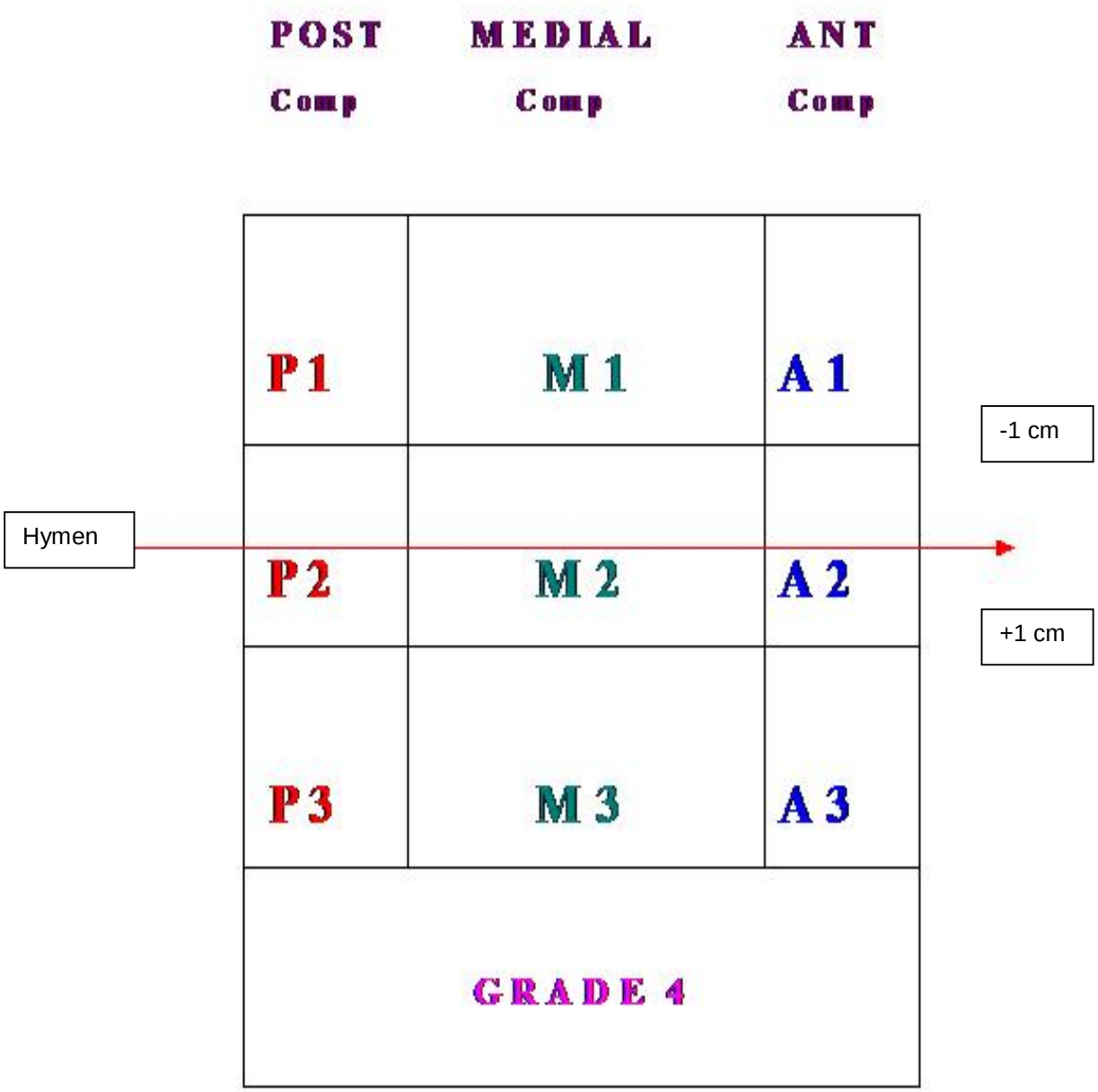

Fig 2.
AMP system
Simplified grading system for prolapse using the hymen as reference landmark.
A= anterior; P= posterior; M= middle

MRI

	A0	A1	A2	A3	A4
A0	**3**	6	3	0	0
A1	5	**6**	1	1	0
A2	3	1	**4**	2	1
A3	0	0	4	**2**	1
A4	1	2	2	1	**2**

^ d Z N T = = P P K P B X = l a Z N V = = P T

h Z = M K P N X = V

	M0	M1	M2	M3	M4
M0	**8**	0	0	0	1
M1	4	**3**	0	0	0
M2	8	5	**3**	0	1
M3	2	5	1	**1**	1
M4	0	1	1	0	**6**

^ d Z O N = Q N K N B X = l a Z O T = R O K

h = Z = M K P

CLINICAL

	P0	P1	P2	P3	P4
P0	**4**	0	2	0	0
P1	4	**1**	4	2	2
P2	5	2	**5**	1	1
P3	0	1	5	**2**	3
P4	1	1	0	0	**5**

^ d Z N T = P P K P B X = l a Z N V = = P T K

h = Z = M K P

Table 1
Comparison of MRI and Clinical grading results. AG = Agree; OD = Over-diagnosed; UD = Under-diagnosed; K = Kappa; CI = Confidence Interval; Pr = Test for symmetry.

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CHAPTER 1

Introduction

1.1 The Levator Ani Muscle

1.1.1 Anatomy of the Levator Ani muscle

The female pelvic floor, is formed of a complex network of muscles, fascia and their nerve supply interconnected to provide adequate support to the pelvic viscera as well as the necessary control of sphincters during defecation and voiding [1]. Injury to the pelvic floor structures through childbirth and hormonal changes associated with ageing in women leads to genital prolapse and both urinary and anal incontinence, significantly affecting women s quality of life [2,3].

The Levator Ani (LA) muscle, is the principal active supporting structure of the pelvic floor and has been traditionally described as consisting of four distinct muscles: the coccigeus, iliococcygeus, pubococcigeus and puborectalis [4]. Since the accounts of Holl in 1897 and Thompson in 1899, the muscle had been divided on comparative anatomical grounds.

Traditionally, the coccigeus derived from the tip and posterior border of the spine is considered to be a degenerating muscle since its function in animals as agitator caude has no real place in humans. It arises from the tip of the ischial spine, alongside the posterior margin of the obturator internus. Its muscle fibres fan out

to be inserted into the side of the coccyx and the lowest piece of the sacrum. The Pubococcygeus included muscle arising form the pubis, either directly from the body or indirectly from the superior ramus via the white line or arcus tendineus. It forms a flat muscle whose fibres are in different functional sets. The bulk of its posterior fibres arising from the white line, fan out backwards in a flat sheet on the surface of the iliococcygeus and are inserted into the tip of the coccyx and the anococcigeal ligament and raphe. The Iliococcygeus muscle includes fibres from the reminder of the white line, and thus indirectly via the attachment of the obturator fascia to the superior ramus of the ilium and from the medial aspect of the ischium. Though the plane between iliococcygeus and coccygeus was clear, the separation of the pubo and iliococcigeus was based on an imaginary line running from the anterior edge of the ischial tuberosity to the junction of the superior pubic ramus with that of the ilium. Lawson s dissection in 1974 [(5)] made a detailed study of the pelvic floor muscles which indicated a subdivision in two functional groups showing a separate plane of the cleavage between muscles which arose from the body of the pubis, and those arising from the white line. Studying muscle insertion he noted that muscles from the pubic body inserted either directly into or provided a sling for the structures intimately associated with the

pelvic viscera forming the pubovisceral (pubococcigeus) component of the LA muscle or vertical component. On the other hand, muscles arising from the white line posteriorly would form a continuous sheet inserting into skeletal structures, mainly lower sacral and coccyx structures. The illeococcigeus muscle would also fan out to insert into the sacrum and coccyx lateral to the sacral foraminae. This second group would be regarded as the diaphragmatic group or horizontal. Recently the traditional view of the LA muscle as a diaphragm has also been challenged by the use of new imaging techniques. Singh et al [(6)], studied the LA muscle using 3D MRI imaging in asymptomatic nulliparous women, and described it as a more functional muscle in two planes with a horizontal and a vertical component. The horizontal part of it had a more visceral supportive role whereas the vertical component (pubo-visceral complex), had a more sphincteric function as it embraced like a sling, segments of the urethra, vagina and rectum.

The LA muscle involvement in providing both pelvic floor support and continence has been extensively described in the literature. As samples for determination of MGF and IGF-I were taken from specific muscles involved in easy or traumatic child birth, it is necessary to describe the location, innervations and function of these muscles from which biopsies were obtained. One of the first

theories contemplating the pelvic floor in a more holistic approach was the integral theory in 1990 by Petros and Ulmsten [7]. In their description of the anatomy, they suggested that any damage to the anterior vaginal wall mid-urethra support structures, mainly the pubo-urethral ligaments, anterior vaginal wall connective tissue and LA muscles, would lead to stress urinary incontinence. In 1992, DeLancey et al [8] described three levels of support within the pelvic floor: level I mainly providing vaginal suspension from the lateral pelvic walls; level II providing attachment to the vagina through the arcus tendineous fascia pelvis; and level III, where the distal vagina is directly attached to the surrounding organs mainly urethra, perineal body and adjacent LA muscle. Above the LA muscle lies the endopelvic fascia [9] described as a network of connective tissue intimately related to the different structures of the pelvic floor responsible for continence. As it will be described later, the LA muscle plays a key role in conjunction with the endopelvic fascia in preventing incontinence and proplapse by preventing constant strain on the ligaments involved [10].

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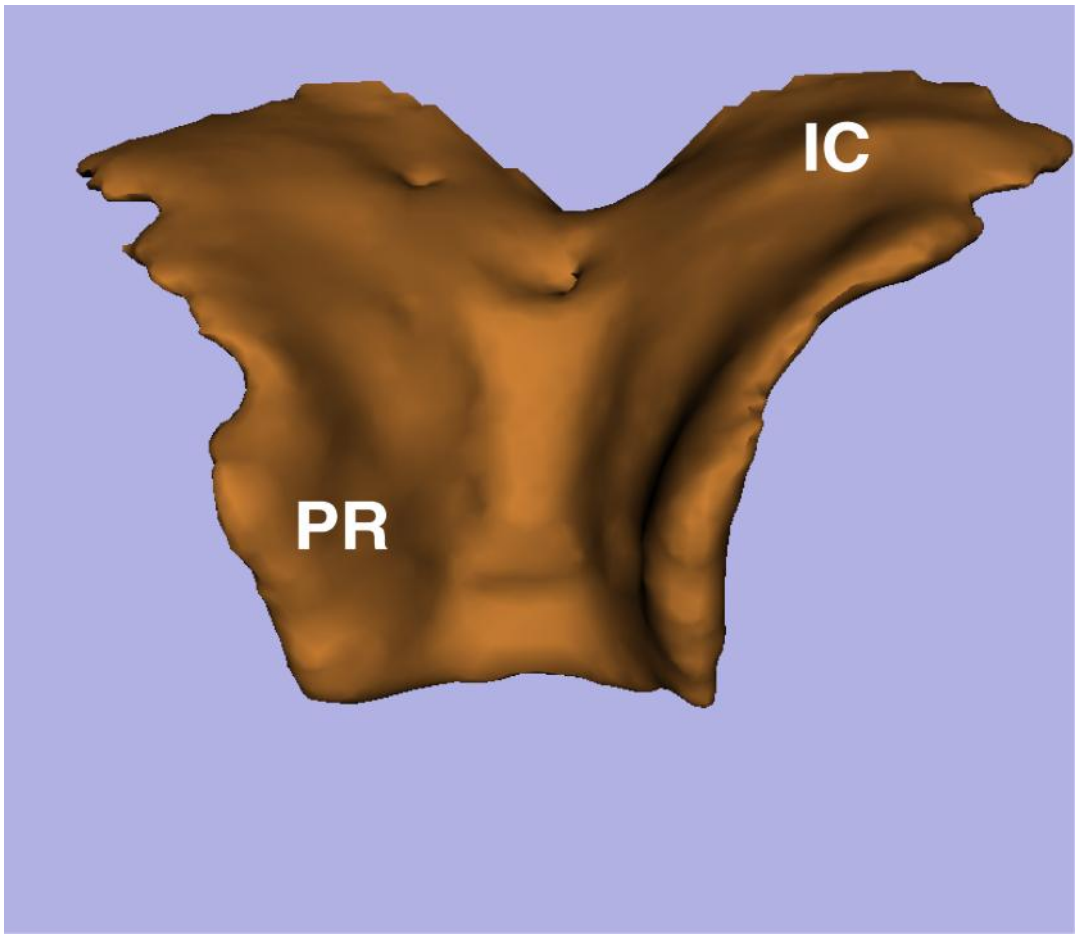


Fig. 3

3D MRI LA muscle image on a nuliparus young woman showing the Illeococcigeus (IC) muscle, horizontal component, and the Pubo-rectalis (PR), as the pubovisceral vertical component[3].

1.1.2 Innervation of the LA muscle

Many well-regarded medical texts and review articles on this subject suggest that the pelvic floor muscles are dually innervated by the pudendal nerve and direct branches of the third and fourth sacral motor nerve roots [11,12]. These suggestions are supported by several post-mortem dissection studies in humans. Lawson et al [13] dissected 13 foetal or neonatal pelvis and found nerves that directly innervated the superior surface of the levator ani muscles that

originated from S3-5 and 2 branches of the pudendal nerve that passed below the pelvic floor along the medial wall of the ischiorectal fossa to supply the under surface of the pubosphinteric (or pubovisceral component) portion of the LA (Fig 4). Sato et al [14] found dissections of 9 adult cadavers (8 male and 1 female) in which the levator ani muscles were innervated primarily by a nerve that originated from the sacral plexus that coursed along the pelvic surface of the pelvic floor muscles and was distributed to the iliococcygeal, pubococcygeal, and puborectalis muscles. However, he also noted that the perineal and inferior rectal branches of the pudendal nerve penetrated the rectal attachments of the puborectalis. In dissections of the pudendal nerve, Shafik et al [15,16] indicated that branches that arose from the inferior rectal nerve penetrated the undersurface of the levator ani muscles. They did not however, examine or comment on any source of innervation from the pelvic side of the muscles. Despite the general acceptance that the levator ani muscles are dually innervated by direct sacral innervations and branches from the pudendal nerve, several studies suggest this may not be the case. After performing detailed dissections of sacral innervation on 3 male cadavers and electrical stimulation studies in 5 patients with neurogenic lower urinary tract dysfunction, Juenemann et al [17]

concluded that the LA muscles were innervated by a nerve that originated from S2-4 that branches at a point proximal to the ischial spine (before the pudendal nerve roots reach the sacrospinous ligament) and then innervates the levator ani muscles on their pelvic surface. No contribution of the pudendal nerves to levator ani innervation was noted. Additionally, many electrophysiological studies have demonstrated that the levator ani muscles and the external anal sphincter have physiologically distinct innervations and that the pudendal nerve does not appear to innervate the iliococcygeal, pubococcygeal, or puborectalis muscles [18,19,20]. In a study conducted by Mathew et al [21], they found that the levator ani muscles were innervated solely by a nerve that originated from S3-5 that travelled along the superior surface (pelvic side) of the muscles before penetrating each of the pelvic floor muscles at their approximate mid point. In none of their 12 dissections conducted in female cadavers, they were able to find a pudendal nerve branch that innervated any of the 3 levator ani muscles, in spite of specific attempts to locate such a contribution. It was concluded that the pudendal nerve innervates the external anal sphincter, the external urethral sphincter, the perineal muscles, and the perineal skin but not the levator ani muscles (Fig 5). This non-pudendal innervation of the LA has been called various names in literature and

textbooks: direct innervation by S2-S4 spinal roots [22], the nerve to the levator ani [23], intrapelvic somatic nerve [24], and several others [25]. Admittedly, small nerve branches can be missed, even in the most careful gross dissections. Thus, this type of study cannot be considered definitive. However, given the consistency of the findings, it is safe to conclude that the major innervation to the iliococcygeal, pubococcygeal, and puborectalis muscles in women is the levator ani nerve, which originates from S3- 5 and travels along the superior surface of the pelvic floor (Fig 6). Any contribution of the pudendal nerve to levator ani innervation appears to be minor.

These findings have been more recently confirmed by Wallner et al [26]. Using a combination of nerve dissection, pudendal nerve blockage simulation, immune-histochemestry and 3D MRI reconstruction they found no significant differences between male and female innervation of the LA muscle. The features they looked at included LA nerve contribution to LA muscle; perineal nerve contribution to LA muscle; and communicating branches between the two (present in 50-60% of individuals). Interestingly, they found instead that differences were present between left and right side of the pelvis and that these followed inter-individual variations.

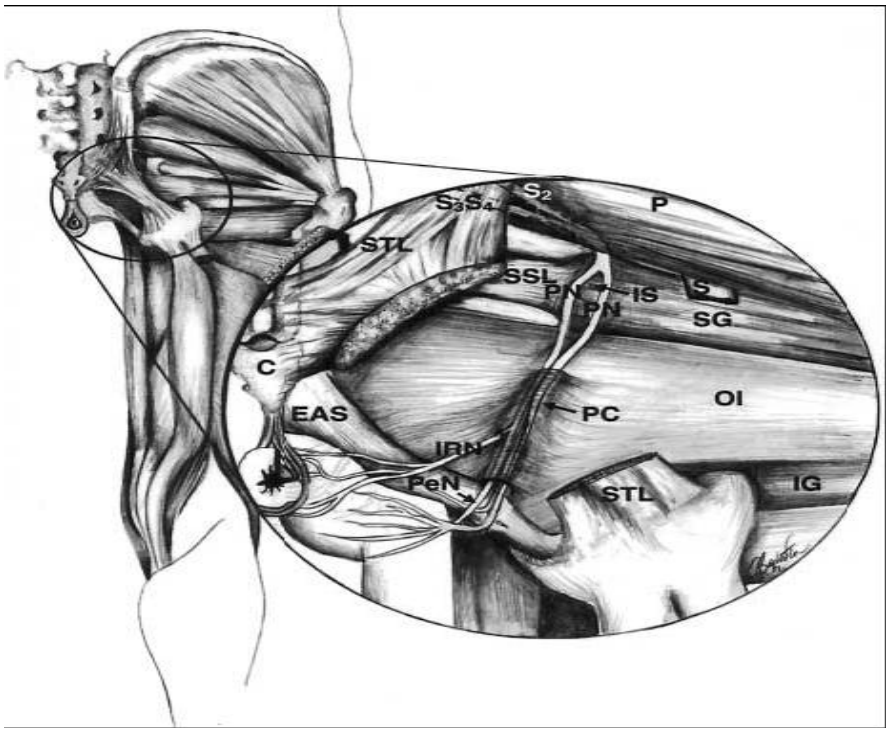


Fig. 4

Course of the pudendal nerve through gluteal region and ischiorectal fossa (transgluteal view). The gluteus maximus and the sacrotuberous ligament have been removed. *S2-S4*, Sacral nerve roots; *P*, piriform muscle*; STL,* sacrotuberous ligament; *SSL*, sacrospinous ligament; *PN*, pudendal nerve; *IS*, ischial spine; *S*, sciatic nerve; *SG*, superior gemellus muscle; *C*, coccyx; *EAS*, external anal sphincter; *PC*, pudendal canal; *OI*, obturator internal muscle; *IRN*, inferior rectal nerve; *PeN*, perineal nerve; *IG*, inferior gemellus muscle. [27]

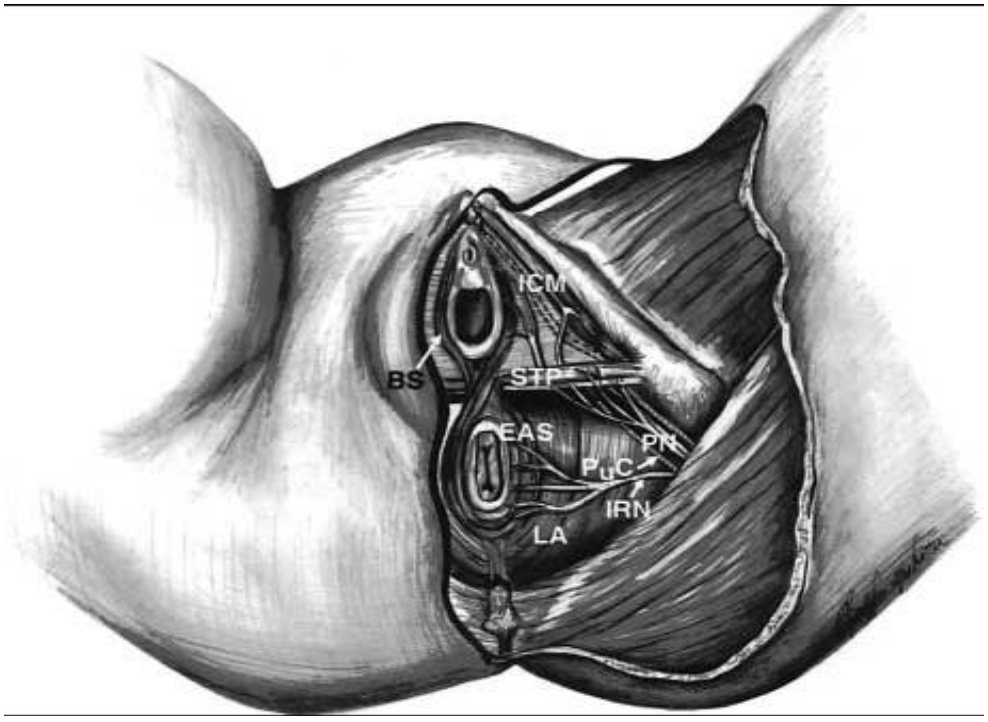


Fig. 5

Terminal branches of the pudendal nerve (perineal view). *IC*, Ischiocavernous muscle; *BS*, bulbocavernous muscle; *STP*, superficial transverse perineal muscle; *EAS*, external anal sphincter; *PC*, pudendal canal; *IRN*, inferior rectal nerve; *LA*, levator ani muscles; *PN*, pudendal nerve [27].

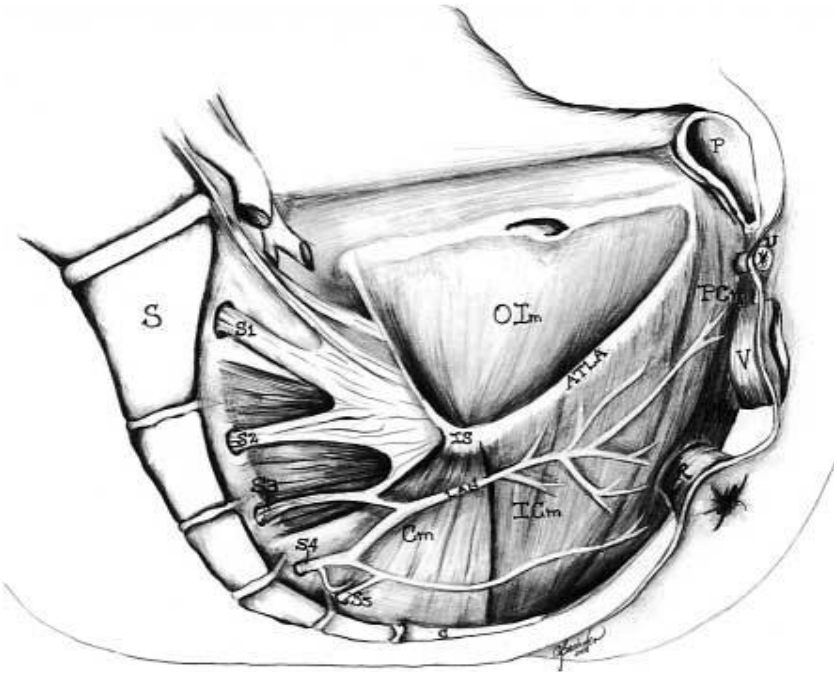


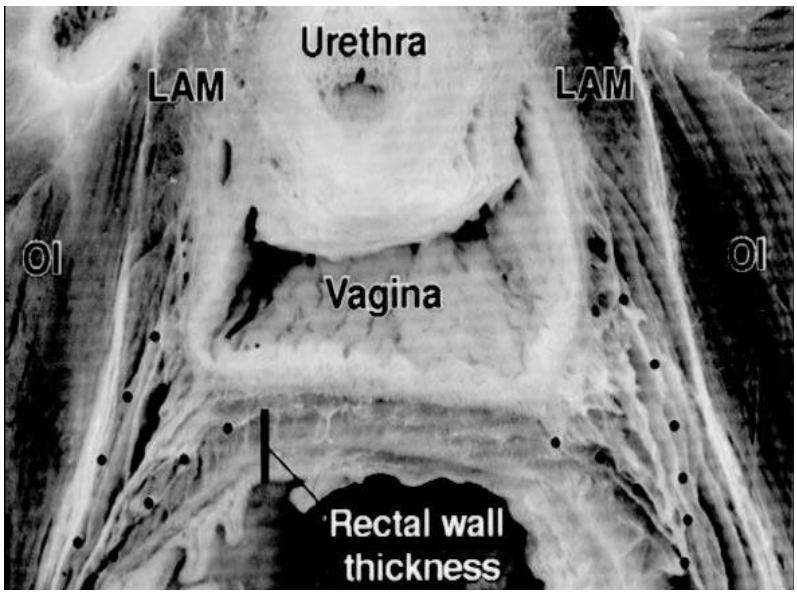
Fig. 6

Illustration of the course of the levator ani nerve (left hemipelvis, sagittal view). *S*, Sacrum; *S1-S5*, sacral foramina; *Cm*, coccygeal muscle; *LAN*, levator ani nerve; *IS*, ischial spine; *ICm*, iliococcygeal muscle; *OIm*, obturator internal muscle; *PCm*, pubococcygeal muscle; *PRm*, puborectal muscle; *ATLA*, arcus tendinous levator ani; *C*, coccyx; *V*, vagina; *U*, urethra; *R*, rectum [27].

1.1.3 The LA muscle in women

The distinction established between a diaphragmatic group and a visceral group acquires particular significance in the female pelvis. As part integrated within the pubovisceral component of the LA muscle, fibres from the pubococcigeal muscle arising more

anteriorly, from the periostium of the body of the pubis, pass well to the sides of the urethra to be inserted to the lateral sulci of the vagina and swing more medially and more inferiorly around the anorectal junction and join with fibres of the opposite side and with the posterior fibres of the deep part of the external anal sphincter [33]. No raphe exists here, and the muscle forms an U-shaped sling which holds the anorectal function angle forwards with no direct bone attachments. This part of the LA has been named as the puborectalis muscle. More medially still, fibres from the U-shaped sling passes behind the vagina into the perineal body forming the pubovaginalis, or sphincter vaginalis. This sling appears as the active support of the female pelvic floor containing within it, the female urethra, lower and upper vagina, and the ano-rectal junction. (Fig 7 and 8)



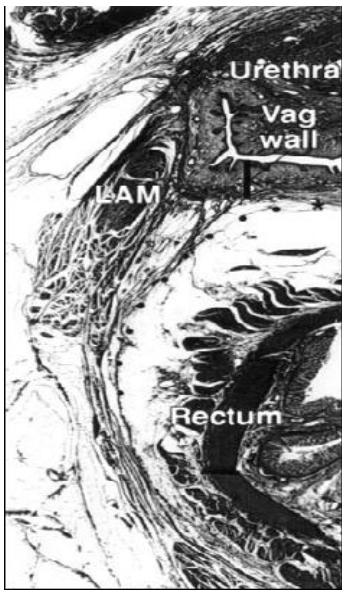


Fig. 7

A, Macroscopic section of 14-year-old nulliparous cadaver showing the Levator ani muscle embracing urethra, vagina and rectum. B, Histologic slide of Mallory trichrome stained section of left half of 1-year-old infant. Note that most fibers of endopelvic fascia (outlined by *dots*) attach to lateral sulcus of posterior vaginal wall *(VAG WALL)* with only a small proportion of fibers connecting with fibers of contralateral side *(asterisk).* In **B** note origin of endopelvic fascia from superior fascia of levator ani muscle *(LAM). OI,* Obturator internus muscle; *URETH,* urethra [32].

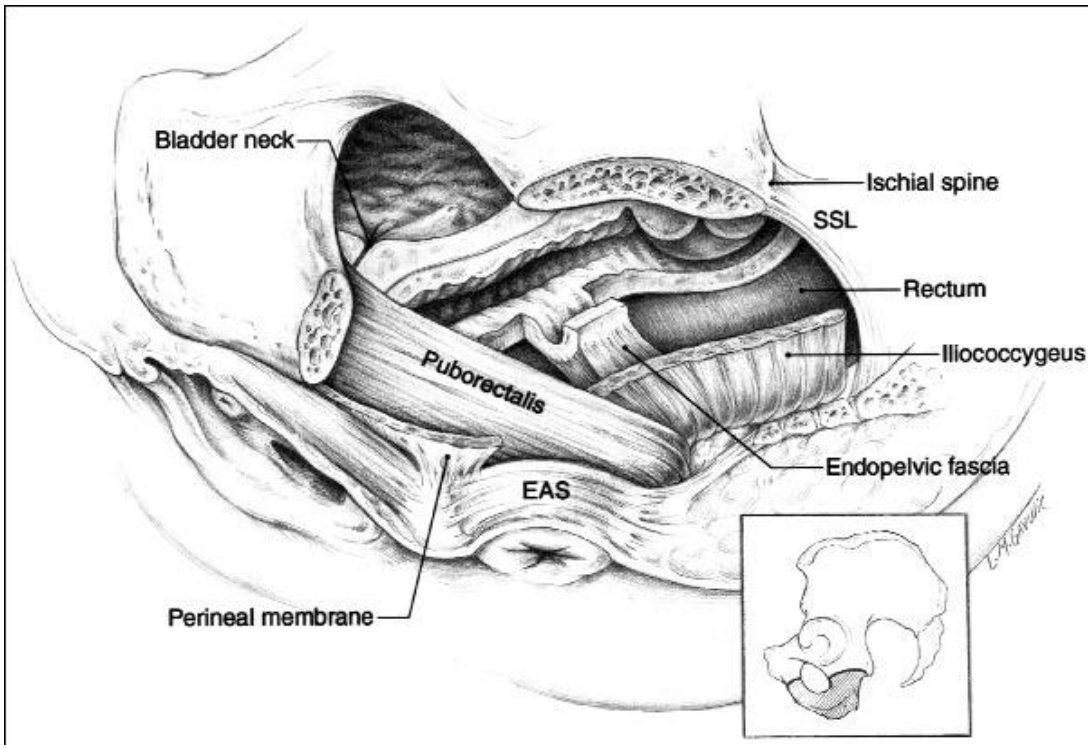
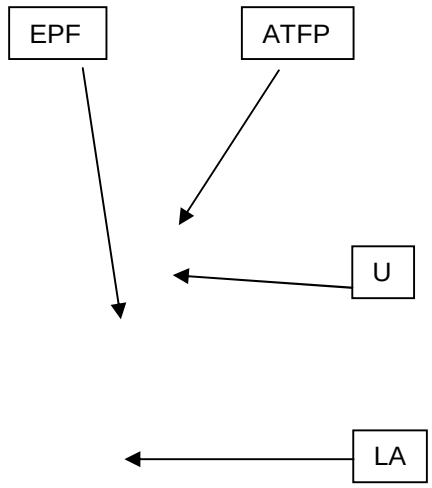


Fig. 8

Lateral view of pelvic organs after removal of left ischial bone and ischial tuberosity. Bladder, vagina, and cervix have been cut in sagittal plane to reveal their lumens. Rectum has been left intact. A strip of posterior and lateral vaginal wall and its attached endopelvic fascia are shown, indicating their position relative to levator ani muscle and this fascia s course and attachment. Both portions of levator ani muscle (puborectalis and iliococcygeus) are visible. Ischial spine and intact sacrospinous ligament *(SSL)* are above level of removed ischial tuberosity. Left half of perineal membrane (urogenital diaphragm) is shown just caudal to puborectalis portion of levator ani muscle after its detachment from inferior pubic ramus that has been removed. *EAS,* External anal sphincter [32].

1.1.4 LA action in women

One of the most distinctive features of the LA muscle is the ability to show a resting myoelectric activity. As demonstrated by Shafik et al[16], levator ani s electromyographic activity would be the result of intraabdominal pressure and visceral weight. Due to its resting myoelectric activity and the U-shape distribution of this muscle, the LA in women maintains direct interaction with the structures involved in providing continence and support to the pelvic floor. Anteriorly, through the Arcus Tendineous Levator fascia, the LA is indirectly involved in the Hammock theory providing urinary continence following strain or a raise of intra-abdominal pressure [29], (Fig 9). The endopelvic fascia in combination with the LA muscle is a main player in this continence mechanism. The integrity of the LA muscle has also been associated with better repair outcome following pelvic floor surgery for anterior compartment prolapse [30].



A **B**

Fig. 9A, 9B

A. Anatomical drawing showing the relationship between the levator ani muscle anterior aspect, arcus tendineous levator fascia, and urethra as contributors to the Hammock theory . The urethra lies on a supportive layer that is composed of the endopelvic fascia and the anterior vaginal wall. This layer gains structural stability through its lateral attachment to the arcus tendineus fascia pelvis and levator ani muscle

B. Closer view of the anatomical relationship between uretra (U), Arcus tendineus fascia pelvis (ATFP), and the LA muscle. The endopelvic fascia (EPF), lies suburethrally extending between both ATFP. Pressure from above compresses the urethra against this hammock-like supportive layer, compressing its lumen closed. The stability of the suburethral layer depends on the intact connection of the vaginal wall and endopelvic fascia to the arcus tendineus fasciae pelvis and levator ani muscles [29].

Attachments of the endopelvic fascia to the vagina have been evaluated in axial and coronal planes. The appearance of the arcus tendineus of the pelvic fascia in axial planes and the direct connection between the lateral vaginal wall and the LA have already been described using MRI [31], (Fig 10). Nonetheless, individual variations from the standard anatomy, were seen in the LA morphology and its anatomical attachments. For example,

in10% of women a visible insertion of the LA inside the pubic bone could not be demonstrated. It is possible that those patients with a lower muscle bulk or weaker urethral supports could be more likely to become symptomatic because of partial defect healing following vaginal birth or as a result of muscle sarcopaenia. However, this assumption can not be made until clear domains of variation have been defined.

Fig. 10a 10b

Axial section at level of middle urethra shows difference in levator ani muscle thickness and configuration. In this, scans from two individuals are compared; scans from one individual are displayed on *left,* and scans from other individual are displayed on *right:* **a,** thin muscle (31-year old nulliparous woman); **b,** thicker muscle (36-year-old nulliparous woman).
Note also that muscle is shaped more like a *V* in **a** and more like a *U* in **b.** *Closed arrowhead,* Right levator ani muscle; *open arrowhead,* insertion of arcus tendineus of fascia pelvis into pubic bone in **b**. *Anatomic variations in the levator ani muscle, endopelvic fascia,and urethra in nulliparas evaluated by magnetic resonance imaging*[29].

In its more middle compartment, through the endopelvic fascia and the hiatus ligament, the LA muscle contributes to the tension needed to support the upper vagina with the uterus and fornices. In its most posterior aspect of the sling, by contracting forward, the LA muscle in combination with the recto-vaginal septum, forms a flap valve mechanism, which provides the upper level of continence of a three levels anal sphincter continence mechanism [32]. (Fig 11)

To summarise, the LA muscle is a unique skeletal muscle with its own specific physiological characteristics, located in an anatomical ly privileged position in order to provide the necessary support to the different pelvic floor organs. At the same time, it is intimately connected with the main structures responsible to provide both urinary and anal continence. Damage occurring to this muscle as a result of physiological over stretch during vaginal delivery may translate into a deficit in organ support and continence function.

Fig. 11

Concept diagram showing mechanics of support and continence of the posterior compartment [32].

A, Closure of pelvic floor by puborectalis muscle *(large arrow)* that compresses posterior vaginal wall against anterior wall. Increases in abdominal pressure result in balanced pressure on anterior and posterior vaginal walls (*arrows*) so that no net force on support results. Caudally, however, there is no balancing pressure, and force results (*dashed arrow*) that must be resisted by the fibres of the perineal membrane (*shaded area*) of perineal body.

B, Absence of levator-mediated closure of pelvic floor. Increases in rectal pressure are unopposed and force on posterior vaginal wall results *(arrow).*

C, Level II supports oppose force shown in **B** *(dashed arrow)* by their upward dorsal tension *(arrows attached to posterior vaginal wall and endopelvic fascia)*.

1.2 Insulin-like Growth Factor 1 (IGF-1)

The existence of the Insulin-like growth factor was first described in 1957 when Salmon and Daughaday[33], described the presence in serum of a new substance in skeletal tissue, through which the Growth hormone (GH) apparently exerted its metabolic effect. This factor was suspected to be growth hormone-induced, since it appeared in the plasma of hypophysectomised rats following administration of growth hormone. One further observation carried by Froesch E et al in 1963[34], described the fact that serum could exert insulin-like growth activity on insulin target tissues such as muscle and adipose tissue. These effects were much greater than expected on the basis of the insulin content of serum. Furthermore these effects were not suppressed by the addition of anti-insulin serum, and were called non-suppressible insulin-like activity (NSILA), possibly closely related to the insulin molecule. In 1972, Pierson and Temin[35], extracted factors from calf serum which stimulate the multiplication of fibroblasts in tissue culture and was termed multiplication-stimulating activity (MSA). Finally, in 1972, it was decided that these three activities represented similar responses and were termed somatomedines [36]

Purification of somatomedine from human serum led to the identification of a neutral peptide of about 7000 Dalton defined as somatomedine A [37,38] and a basic peptid termed somatomedine C [39]. Both substances were found to be under growth hormone control.

Analysis of NSILAs from human serum resulted in the first elucidation of the primary structures of two peptides that were termed human IGF-I [40] and human IGF-II [41]. Purification and analysis of MSAs in the serum free medium conditioned by the Buffalo rat liver cell line led to structural characterization as the rat counterpart of human IGF-II[42]. Sequence analysis of the somatomedine C confirmed that it was identical in structure with IGF-I[43]. Somatomedine A was shown to represent a mixture of IGF-I and IGF-II [44]. In 1987, to prevent confusing the nomenclature, IGF-I was described as a 70 amino acid residue single chain peptide structurally related to human proinsulin, and IGF-II as a 67 residue neutral peptide that is structurally similar to IGF-I, but much less GH-dependent.[45,46,47]

Although initially Insulin-like growth factor (IGF-I) was thought to be produced mainly by the liver and induced by GH, as a main systemic regulator of tissue mass during post-natal growth, more recent studies have found it to be produced by several other

tissues including skeletal and cardiac muscle. It has also been documented to be involved in tissue maintenance and prevention of cell death, as well as being responsible for regulation of protein synthesis and activation of muscle satellite cells (stem cells) that are required for muscle repair [48]. (Fig 12)

Fig. 12

A; Diagram showing of interaction of GH and IGF-1 in the liver as a post-mitotic growth agent. B; IGF-1 has a local action within the muscle following splicing into MGF and IGF-1a.

1.2.1 Satellite (progenitor) cells in muscle

Satellite cells are small mononucleate precursor cells that are located between the basal lamina and sarcolemma of muscle fibres (Fig 13). These cells are believed to remain mitotically inactive until the muscle is subjected to increased mechanical loading or damage. It is believed that these cells proliferate and differentiate during the first stage but do not initially enter the myogenic pathway. The second stage is initiated by IGF-1 which induces them to fuse with existing fibres, providing extra nuclei to maintain and supplement the DNA. These two phases seem to be the response of different IGF-1 splice variants, each with specific actions [49,50], and may be involving different receptors [51].

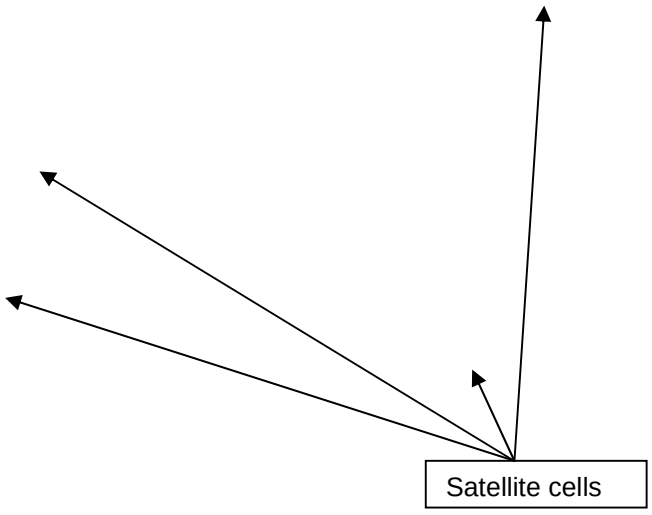


Fig. 13

Picture showing different sections of skeletal muscle fibers where the satellite cells can be seen within the extracellular matrix.

1.2.2 IGF-1 receptor

The cellular effects of IGF-I are mediated by the activation of a specific tyrosine kinase receptor. IGF-I binds with high affinity to the type 1 dimeric receptor, which is structurally and functionally homologous to the insulin receptor. It is composed of two ligand binding subunits and two transmembrane subunits. It is known that the biological activity of a hormone or growth factor does not simply reflect its levels, but it also depends on the abundance of the receptor and the affinity of its interaction.

1.2.3 IGF-binding proteins (IGFBP)

Pre-receptor regulation of IGF-1 is also important in determining its activity. Six binding proteins have been characterised to date acting as modulators of IGF-I action. Their main functions are to 1) stabilize and transport IGFs from the circulation to the peripheral tissues, 2) maintain a reservoir of IGF in the circulation, 3) reinforce or inhibit IGF action, and 4) mediate IGF-independent biological effects. Whilst IGFBP-3 has been shown to be responsible for maintaining IGF-I levels in the circulation in conjunction with another protein called acid labile subunit, variations in expression of

IGFBP-4 and IGFBP-5 in muscle may play a significant role in muscle adaptation to loading [52].

1.2.4 Expression of locally produced growth factors following mechanical activity.

Physical activity seems to be mainly responsible for changes in expression of two IGF-1 main splice variants seen in muscle, although it has only recently been appreciated that there are actually six types of IGF-1. De Vol et al [53] demonstrated that there was a threefold increase in total IGF-I mRNA levels in the soleus and plantaris muscle in 11 to 12 week old female rats after tenotomy induced hypertrophy. These levels were maintained 2, 4, and 8 days after the experiment, thereby suggesting that there was a relationship between muscle growth and local gene expression of IGF-I. DeVol *et al.* [54] also suggested that during muscle growth, locally produced IGF-1 had two distinct functions with respect to satellite cell involvement in that it stimulated both proliferation and differentiation of satellite cells. The role of IGF-1 in satellite cell activation was further supported by experiments in which direct infusion of IGF-1 into the tibialis anterior muscle of adult rats resulted in an increased total muscle protein and DNA content, demonstrating skeletal muscle hypertrophy related to satellite cell

activation [55]. Further evidence for the role of these cells in hypertrophy is provided by an experimental approach whereby satellite (progenitor) cells were prevented from proliferating after treatment with [gamma]-radiation. The subsequent overloading of the adult rat soleus muscle failed to evoke hypertrophy of the overloaded irradiated muscles [56].

The cDNA of two IGF-I splice variants expressed in muscle (IGF-1c and IGF-1a) have been cloned in rat and human [57]. From these, two main isoforms are expressed by muscle when it is subjected to mechanical stimulation leading to a process of alternative splicing by which exons are arranged in different combinations from pre-mRNA. Although initially, it was believed that splicing of IGF-1 would mainly respond to mechanical damage, a study conducted by Hill and Goldspink [58], have suggested that the same splicing occurs following other types of cellular damage.

Yang et al [57], showed that it was the splice variant IGF-1c which appeared markedly upregulated in rabbit muscle following acute limb stretch. In the same study it was shown as well that expression of the systemic IGF-IEa isoform was also induced by mechanical stimulation. Later studies using electrically stimulated muscle compared to mechanically stretched muscle, supported these findings as expression of IGF-1Ec and IGF-IEa were not

significantly increased in the electrically stimulated group when compared with muscle from sham-operated controls. [59]. The expression of both systemic and autocrine IGF-I in muscle provides an interesting link between the mechanical signal and a marked increase in structural gene expression involved in tissue remodelling and repair.

The IGF-I gene contains two reader sequences or start sites at the exons 1 and 2. Transcripts initiating at exon 2 are common in the liver and are highly GH dependent, whereas transcripts initiating at exon 1 are widely expressed in extrahepatic tissues although these tissues also express class 2 IGF-1. Alternative splicing of the gene IGF-I generates three different E peptides that have a common N terminal sequences but alternative C-terminal sequences. These have been described as IGF-IEa, IGF-IEb, and IGF-IEc and may be involved in different functions depending on the species and non-hepatic tissues in which their expression is regulated by hormones. A schematic representation can be seen in figure 14.

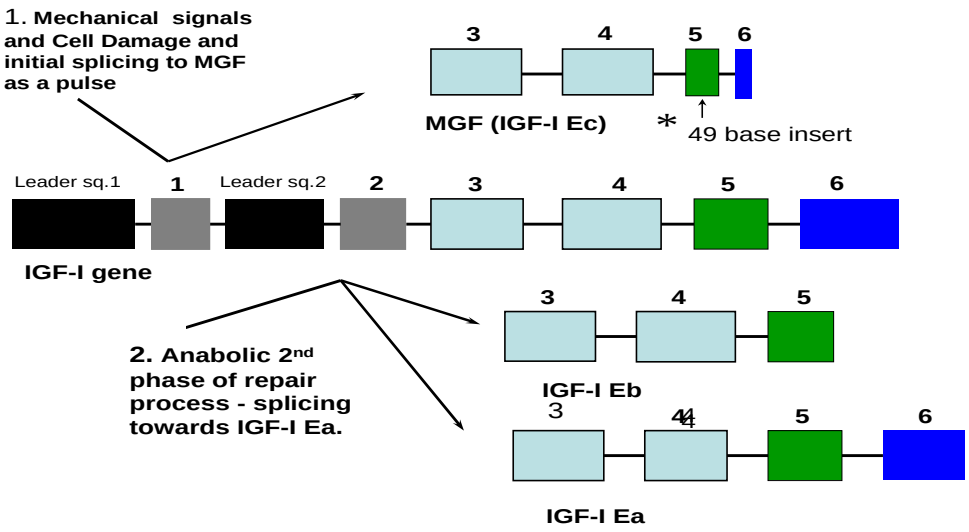


Fig. 14

Schematic representation of the IGF-1 splice variants. The terminology used here is for human IGF variants. Human MGF (=IGF-1EC) corresponds to rat IGF-1Eb

The first isoform is similar to the hepatic endocrine type of IGF-I and has been named as IGF-IEa [(60)]. Transgenic mice that over express this particular isoform in skeletal muscle have been shown to have pronounced muscle hypertrophy [(61)], and it seemed that the older animals showed signs of protection against the normal loss of muscle mass associated with aging. The second isoform, IGF-IEb in humans, although predominantly expressed in the liver, its role in muscle is not yet clear. The third isoform is a splice variant resulting from a novel splice acceptor site in the intron preceding exon 6 and is thought to be generated within muscle when subjected to stretch and overload [(58)]. Because of a confusion in the nomenclature in the rat vs. the human, this isoform was named mechano growth factor

(MGF), and corresponds to the IGF-IEc isoform in humans and IGF-IEb in rats. Structurally, it differs from the liver types because it has 49 base pairs (52 in the rat) insert which is responsible for a reading frame shift resulting in a unique E domain sequence. It also seems not to be glycosylated, therefore it would be expected to have a shorter half-life than the liver s counterparts, which suggests an autocrine/paracrine mode of action rather than a systemic one, by binding to a muscle-specific binding protein embedded in the interstitial tissue spaces. It is precisely there, where it would have its specific action by stimulating division of mononucleated myoblasts (satellite cells) and increasing the number available to fuse to form syncitial myotubes which become innervated and develop into muscle fibres. More recently, in a study conducted by Kandalla et al, a terminal 24aa E peptide within MGF has been identified as the peptide directly involved in satellite cell activation, proliferation and fusion for muscle repair. Following muscle damage during the post-mitotic (post natal) phase, it would seem that this specific sequence within MGF would promote activation and differentiation of additional stem cells to fuse with damaged muscle fibres [63]. This initial activation of muscle satellite (stem) cells [64], is important as muscle is a post-mitotic tissue. The activated satellite cells will then fuse with the muscle fibres to

provide the extra nuclei for muscle fibre repair as well as hypertrophy. Later, the IGF-1 gene is spliced to the isoform IGF-1Ea, which recent studies suggest provides the main anabolic response in generally up-regulating protein synthesis [65]. (Fig. 15) The time course of these splice variants following muscle injury has also been studied in vivo and in vitro. These studies have shown a peak for MGF and IGF-1Ea, 24 hours and 72 hours post exercise respectively [66,67].

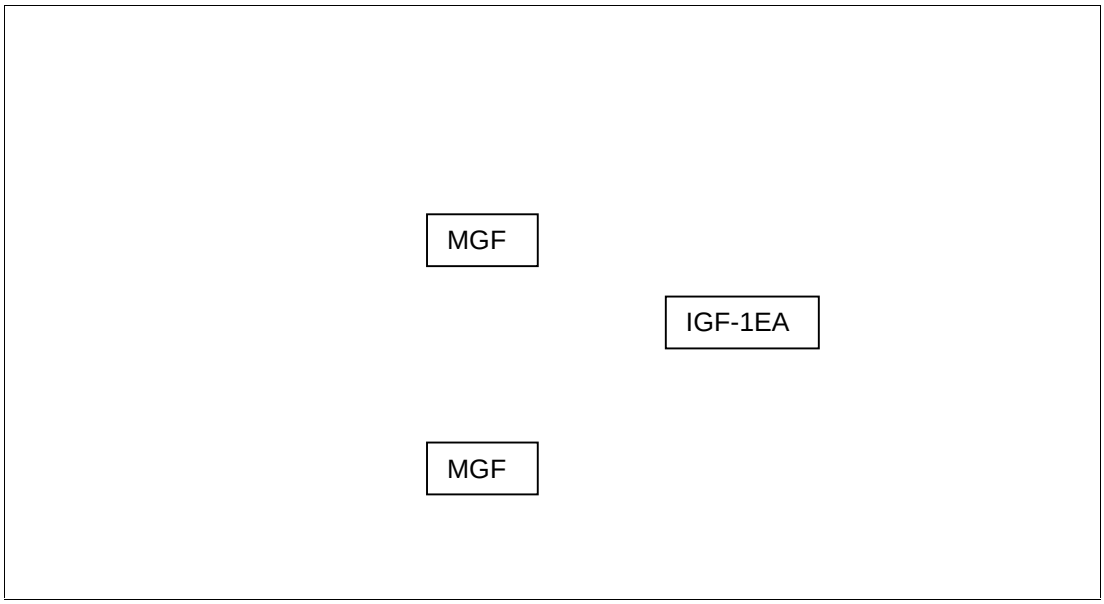


Fig. 15

Picture representing the combined action of MGF on the satellite cells followed by the anabolic input provided by IGF-1Ea

Studies conducted on tissues following injection of the myotoxic agent bupivacaine, suggest that MGF would have a dual action in that, like the other IGF-I isoforms, it up regulates protein synthesis

as well as activating satellite cells [64]. It has been shown that MGF is a potent inducer of muscle hypertrophy in experiments in which the cDNA of MGF was inserted into a plasmid vector and introduced by intramuscular injection. This resulted in a 20% increase in the weight of the injected muscle within 2 weeks, and the analyses showed that this was due to an increase in the size of the muscle fibres [68]. Similar experiments by other groups have also been carried out using a viral construct containing the liver type of IGF-I resulting in a 25% increase in muscle mass over a 4 months period [69]. Hence in its dual role, MGF not only would induce satellite cell activation but also promote protein synthesis suggesting it is a much more potent inducer of rapid hypertrophy than the liver type or IGF-IEa.

1.2.5 MGF Activation

MGF-24aa-E peptide has been identified as the main peptide acting as precursor of muscle proliferation and repair, however which proteins or messengers could be involved in MGF stimulation and its expression has has remained elusive to investigators and only recently documented. It is widely accepted that MGF upregulation results from mechanical stretch and tissue damage. In a recent study by Kravchenco et al [70], three myofibrillar proteins, identified

by immunoblotting as myomesin, myosin binding protein C, and titin, have shown the capability of stimulating MGF synthesis. Activation of MGF was associated with an increase of intracellular cAMP whereas its inhibition arrested further activation of MGF.

1.2.6 MGF as a neuroprotective agent

Early studies in animals published in 2004 using IGF-1Eb cDNA (the rabbit equivalent of MGF), was injected into rats facial nerve. A month later following avulsion of the muscle, the rats injected were protected from motoneuron loss [71]. More recent studies injecting the same plasmids in a model mimicking amyotrophic lateral sclerosis seem to yield similar results [72]. Studies have also been conducted in brain cells following ischemic injury. Administration of a synthetic MGF peptide following reperfusion of the ischemic tissues was associated with increased neuronal survival [67]. Finally, synthetic MGF has also shown to be protective against neurotoxin-induced stress and apoptosis. Rats receiving MGF infusion were protected from motor behaviour deficits and neuronal cell death in dopamine neurons within the substancia nigra [73]. The mechanism explaining this process seems to be protein kinase C dependent and has been recently described by Quesada et al.[74,75]

1.3 Muscle physiology

Muscle organs consist of individual muscle fibres (=muscle cells). The fibre consists of parallel packets of many myofibrils, and each myofibril is a linear arrangement of sarcomeres. The sarcomere is the basic contractile unit of muscle. Within the sarcomere two proteins, myosin and actin, are precisely arranged resulting in the striated appearance of cardiac and skeletal muscle when viewed under a microscope. Most of our muscle mass is skeletal muscle. (Fig 16)

Like nerves, the muscle fibre is an excitable tissue that generates an action potential (AP). The AP of skeletal muscle involves activation of voltage-dependent Na+ channels which cause release of intracellular Ca++ from the sarcoplasmic reticulum (SR), a unique organelle of skeletal and cardiac muscle. Ca++ through a series of events causes actin to repeatedly advance over the myosin (=cross-bridge cycling) resulting in shortening of the sarcomere and contraction of the muscle fibre (=sliding filament theory of muscle contraction). During each cross-bridge formation ATP is used [76].

The skeletal muscle organ contracts to varying degrees. This is possible because a number muscle fibres are innervated by a

single neuron (called a motor unit). The number of motor units is much higher in small muscles such as the extraocular muscles that are responsible for precise delicate movements whereas in the limb muscles most fibres are activated by one motoneuron. Also some motor units have lower thresholds than others so it is possible to sum individual motor units to get graded muscle contraction.

Muscle fibres also differ functionally. Type I (=Slow-oxidative) fibres are slow contracting, have high oxidative capacity, contain many mitochondria, and are richly vascularised. These fibres are common in muscles where long term muscle use is important (e.g., marathon running). In small animals, type IIb (=Fast-glycolytic) fibres are fast contracting, utilize anaerobic glycolysis, have few mitochondria, and are poorly vascularised, but have great intensity of contraction. It was realised that the type IIb fibres are in fact too fast for the muscles of larger animals and a slower contracting myosin is found in larger animals including humans [77]. The human type IIc are found in muscles where strength is needed for a short time (e.g. sprinter). A third fibre type, Type IIa (Fast-oxidative), has intermediate properties. Skeletal muscle is capable of contracting under anaerobic conditions due to a number of adaptations (creatine phosphate stores, myoglobin, lactic acid build-up, etc.)

A number of factors influence skeletal muscle mass, such as exercise and hormones (Growth Hormone and Testosterone)[78]. The effect of exercise on muscular development is more apparent in younger individuals. This development is possible because of an abundance of special stem cells, the satellite cells. Satellite cells as already discussed, are also involved in muscle repair. Although availability of satellite cells decreases with age in animal studies, the same decline in satellite cell numbers has not been seen in human muscle with increasing age [79, 80]. In addition, humans fail to demonstrate a difference in the proliferative potential of satellite cells derived from children aged 9 years and those isolated from adults greater than 60 years of age [81]. Thus, it is apparent that the impaired skeletal muscle regenerative response seen with ageing in humans likely results from more factors than just declining satellite cell numbers and proliferative capacity.

Lack of use and/or age result in atrophy of skeletal muscle (sarcopenia). These age effects (e.g., increased frailty) are often more apparent in women than men because women generally have less muscle mass than men [82].

Fig. 16

The sarcomere is the basic contractile unit of muscle. Within the sarcomere two proteins, Myosin and Actin, are precisely arranged resulting in the striated appearance of cardiac and skeletal muscle. Re-distribution of internal strain within myofibrils and muscle fibres may be a key player, particularly, during stretch or relaxation so that force kinetics parameters are strongly affected by sarcomere dynamics

1.3.1 Eccentric damage

In eccentric damage [81] the contracting muscle is forcibly lengthened. It is generally agreed that there are two prominent signs of damage in a muscle immediately after it has been subjected to a series of eccentric contractions. These are the presence of disrupted sarcomeres in myofibrils and damage to the excitation contraction (E C) coupling system. It remains a point of controversy which of these two represents the primary event.

There is a specific hypothesis for the process of sarcomere disruption (Fig. 17A,17B). It has been known for some time that the descending limb of the sarcomere length tension curve is a region

where sarcomere inhomogeneities develop [83,84]. It has been proposed by Morgan et al (1990) [85] that during active stretch of a muscle, most of the length change will be taken up by the weakest sarcomeres in myofibrils or, more strictly speaking, the weakest half-sarcomeres. On the descending limb of the length tension curve, these sarcomeres will become progressively weaker and when they reach their yield point, they will lengthen rapidly, uncontrollably, to a point of no myofilament overlap, where tension in passive structures balances the active tension in adjacent sarcomeres that still have myofilament overlap (Fig 18). This process is reiteratively repeated, with the next-weakest sarcomere stretching, and so on. It is postulated that overstretched sarcomeres are distributed at random along muscle fibres. At the end of the stretch, when the muscle relaxes, myofilaments in the majority of overstretched sarcomeres re-interdigitate so that they are able to resume their normal function. A few may fail to do so and become disrupted [86]. During repeated eccentric contractions it is postulated that the number of disrupted sarcomeres grows, until a point is reached where membrane damage occurs. It is at this point that damage to elements of the E C coupling machinery becomes apparent. Subsequently the fibre may die (Fig. 19).

The sarcomere inhomogeneities hypothesis predicts that damage will only occur if sarcomeres are actively stretched to beyond optimum length. If sarcomere disruption and damage are especially prevalent on the descending limb of the muscle s length tension curve, indicators of damage should show length dependence. In previous studies conducted in both rat and toad muscle the shift in optimum length and the fall in active tension post-contraction depended on the length range over which stretches were given [87,88]. When the muscle relaxed, some overextended sarcomeres did not re-interdigitate [85] and this meant that neighbouring sarcomeres returned to a shorter length than before the contractions. The muscle would therefore have to be stretched further before passive tension in these shorter sarcomeres reach to measurable levels. Other changes after strenuous eccentric exercise, a fall in active tension, shift in optimum length for active tension, and rise in passive tension, are seen, on balance, to favour sarcomere disruption as the starting point for the damage.

As well as damage to muscle fibres there is evidence of disturbance of muscle sense organs and of proprioception. The current view of the mechanism is that the tissue breakdown products sensitise nociceptors so that these respond to stimuli that are normally non-noxious. Hence the muscle is tender to local

palpation, stretch and contraction. It has recently been proposed that a component of the delayed soreness from eccentric exercise involves large-fibre mechanoreceptors [89,90]

A B

Fig. 17A 17B

A. Sarcomere inhomogeneities theory: sarcomeres fail to interdigitate following overextension. This is followed by damage to the excitation contraction coupling (E-C) system.

B. Postulated distribution of elastic filaments in sarcomeres. The top two diagrams consider a sarcomere with elastic filaments only linking the ends of the thick filaments to the Z-lines. The numbers indicate the distribution of tension. Total tension is set at 80 % of maximum to indicate that the sarcomere is on the descending limb of its length-tension relation. When one half-sarcomere becomes over-stretched, as a result of an eccentric contraction, tension borne by its myofilaments drops to zero and the full tension is borne by the elastic filament. The other half-sarcomere is unaffected since its isometric tension capability remains the same. When a second elastic element is included in the model, one which spans the full length of the sarcomere (lower 3 diagrams), overstretch of one half-sarcomere leads 50 % of the tension to be distributed to the elastic element in series with the thick filaments while, because of its proportionately smaller extension, that spanning the sarcomere bears less (30 %). It will also contribute 30 % of the tension to the other half-sarcomere, the remaining 50 % being distributed between the series elastic element (5%) and the cross-bridges (45 %). Since this half-sarcomere's isometric tension capability remains at 70 %, it shortens until tension in the series element has fallen to zero and myofilament overlap is somewhere on the ascending limb, generating 60 % of the tension, leaving 20 % in the elastic element spanning the sarcomere. This is what is observed under the electron microscope, one half-sarcomere over-stretched to beyond overlap, the other half very short. This kind of model indicates that passive tension in the whole sarcomere becomes significant when one half becomes over-stretched [85].

Fig. 18

Changes in mechanical properties of muscle following a series of eccentric contractions

A. Disruption of sarcomeres. Computer simulated sarcomere length-tension relations. The dashed line is the active length-tension relation taken from Gordon, A.M., *et al* (1966). *The variation in isometric tension with sarcomere length in vertebrate muscle fibres. Journal of Physiology* ***184****, 170 192*. The dotted line is an exponential curve representing passive tension; the continuous line is the total tension. Tension is normalised relative to the maximum active tension. Length is given as that of a postulated muscle fibre comprising 10 000 sarcomeres with a sarcomere length of 2.5 m at optimum length. The control curve is the continuous curve on the left. After a series of eccentric contractions 10 % of the sarcomeres have their active force set to zero to simulate becoming disrupted, leading to a shift in optimum length of the total tension curve by 3 mm (continuous curve to the right).

B. Adaptation of the muscle fibre following injury from eccentric exercise. The continuous curve is the control total tension curve as in the upper panel, the dashed curve, that after the number of sarcomeres in series has been increased by 10 %, without changing the length of the tendon. It has led to an increase in optimum length by 2 mm (85).

Fig. 19

Postulated series of events leading to muscle damage from eccentric exercise
During an active lengthening, longer, weaker sarcomeres are stretched onto the descending limb of their length tension relation where they lengthen rapidly, uncontrollably, until they are beyond myofilament overlap and tension in passive structures has halted further lengthening. Repeated overextension of sarcomeres leads to their disruption. Muscle fibres with disrupted sarcomeres in series with still functioning sarcomeres show a shift in optimum length for tension in the direction of longer muscle lengths. When the region of disruption is large enough it leads to membrane damage. This could be envisaged as a two-stage process, beginning with tearing of t-tubules. It would be followed by damage to the sarcoplasmic reticulum, uncontrolled Ca2+ release from its stores and triggering of a local injury contracture. That, in turn, would raise muscle passive tension. If the damage was extensive enough, parts of the fibre, or the whole fibre, would die. Breakdown products of dead and dying cells would lead to a local inflammatory response associated with tissue oedema and soreness [85].

1.3.2 Skeletal muscle and age

The rate at which muscle protein is synthesized is reduced in later life. Balagopal *et al.* [91] showed that whole body protein synthesis was 19% lower in older individuals (77 2 yrs) when compared with younger individuals (23 1 yr). More specifically, muscle protein synthesis was 55% lower, whereas synthesis rates of nonmuscle proteins remained the same. This was in general

agreement with the results of an earlier study by Welle *et al.* [92] who showed that the fractional rate of myofibrillar protein synthesis was 28% lower in older (62 81 yrs) compared with younger (21 31 yrs) men. In addition to overall changes in protein synthesis, there are a number of structural and compositional differences that are characteristic of the ageing muscle. A loss of contractile proteins relative to other cell components and increased amounts of connective tissue and intramuscular fat have been reported. Whether there is a change in the relative distribution of the main histochemically determined fibre types with ageing remains somewhat unclear, although it is certain that there are less fast myosin heavy chain isoforms (in part due to the atrophy of the faster Type II fibres).

The effects of the aging process on the local regulation of the IGF-I isoforms and its coupling to satellite cell activation has yet to be fully determined. Hameed et al [93] and Owino et at [94], following demonstration of IGF-I splice variants in humans, concluded that their expression seemed to be impaired in the elderly. If these changes seen in skeletal muscle, replicate in the LA muscle of elderly women it may contribute to explain the late appearance of symptoms after menopause and the clinically seen increased

failure of conservative therapies (pelvic floor exercises) aiming at reinforcing the continence properties of the LA [95].

1.4 Pelvic floor damage following vaginal delivery

The relationship between vaginal delivery and its effect on the pelvic floor has been widely acknowledged in clinical practice and reported throughout the literature [96,97,98,99]. Studies so far have covered most of the aspects associated with pelvic floor dysfunction and its analysis, including histo-morphological changes in the LA muscle and attached fascia[100], collagen variations[101,102], imaging[103], and neurophysiological performance pre and post delivery[104-107] Fig (20A, 20B). It is also accepted that impairment of the LA as an active support of the pelvic floor leads to progressive mechanical stress on passive supports[108]. Therefore, anatomical or physiological alterations within the LA muscle and/or the endopelvic fascia will effectively lead, in the short and/or longer term, to permanent damage of the female pelvic floor by the means of urinary or anal incontinence, and with or without genitourinary prolapse. More recently the traditional view of the LA muscle as a diaphragm has been challenged by the use of new imaging techniques. Singh et al [109], studied the LA using 3D MRI imaging on asymptomatic nulliparous women, and described it as a two

planes muscle with a horizontal and a vertical component. Histomorphological assessment on the LA muscle performed by Shafik et al [110] on 45 premenopausal unfixed and fresh female cadavers (nulliparous and parous), revealed that relationship between type I (slow twitch) and type II (fast twitch) fibres was 66% and 34% respectively, regardless of age, parity, and location of the biopsy. When comparing nulliparous and parous women they found that vaginal delivery led to significant increases in morphological markers of muscle damage including centrally located nuclei, fibrosis, and variation of fibre diameter. These findings resulting from damage, were also seen in nulliparous women with increasing age whereas no further increase of these changes were seen with ageing in women with previous vaginal deliveries. They were not able to show either, any evidence of neurogenic damage neither in parous women nor in women over 40 years of age. In agreement with previous evidence this suggests that most of the neurological damage observed following vaginal delivery is restored within two months post-delivery [104]. Nevertheless, there is still disagreement in this area when looking at pathological findings in relation to incontinence. The singularity of the LA muscle has also been highlighted as it is one of the human muscles known to have a resting myoelectric activity, probably as a result of the physiological

response to visceral weight and intra-abdominal pressure supported. Shafik et al [110] compared histological sections of 8 adult cadavers and 4 fully mature neonatal deaths, showing the existence of smooth muscle in the more medial aspect of the LA and striated skeletal muscle in the more perineal aspect of it, in consonance with the previously described two functional aspects of the LA. Nevertheless, this smooth muscle seen in adults was noted to be absent in neonates, suggesting then a possible adaptation process related to ageing and the forces mentioned before. Additionally, Copas et al [111], looked at the expression of oestrogen receptors in LA and fascia in women undergoing gynaecological surgery and showed negative staining in the LA for both asymptomatic or symptomatic patients.

Fig. 20A 20B

A. Real picture of head crowning during second stage of labour (pushing phase). It is at this point when maximum distension of the pelvic floor structures takes place.

B. Pelvic view representing the pelvic floor structures at risk of injury during vaginal delivery. The LA muscle is represented in this picture as part of the pelvic floor muscles. Illustration of Vaginal Childbirth and Pelvic Damage from the Netter Collection. www.netterimages.com (Image ID 20252)

1.4.1. Pathophysiology of the levator ani muscle during vaginal delivery.

In a study conducted by Kuo Cheng-Lien et al [112], a three dimensional model was designed to asses LA stretch during vaginal delivery. The structural model of the LA muscle related passive tissues was constructed using serial magnetic resonance images from a healthy nulliparous 34-year-old woman, published anatomic data, and engineering graphics software. The model was

used to quantify pelvic floor muscle stretch induced during the second stage of labour as a model fetal head progressively engaged and then stretched the iliococcygeus, pubococcygeus, and puborectalis muscles (Fig 21).

The maximum pubococcygeal (PC) muscle stretch ratio of 3.26 found in this study exceeds by 217% the largest non-injurious stretch (1.5 stretch ratio, upper limit of shaded region), (Fig 22), observed in nongravida passive striated appendicular muscle. If injury is caused by stretch exceeding a maximal permissible value, the authors concluded that the most medial pubococcygeus (pubo-visceral component) muscle is at a greater risk for injury than any other component of the levator ani muscle during the second stage of labour. The fact that abnormalities are indeed observed in this very part of the levator ani muscle several months postpartum supports the hypothesis of a stretch-related injury mechanism, although they could not exclude the possibility of other injury modes. It also provides validation of model results relative to clinical observations.

The most medial portion of the pubococcygeal muscle also attaches to the connective tissue of the perineal body. At this point in time, it is not certain whether the stretch occurs solely in the pubococcygeal muscle itself, solely in the passive tissues of the

perineal body to which it connects caudally, or in both structures in similar or differing proportions. But irrespective of the stretch distribution within the medial pubococcygeus perineal body complex, the total stretch ratio of the combined structures must reach 3.26 for the fetal head to be delivered. Since this pioneering work was conducted, other models looking at the elasticity of the tissues involved during birth, simulation of injury, or positioning of the head have also tried to reproduce the physiological strain inflicted to the LA muscle concurring that vaginal delivery can markedly affect the anatomy and function of the LA muscle specifically in the posteriomedial aspect of the puborectalis muscle where a maximum stretch ratio of 3.5 to 1 took place[113, 114, 115]. The use of forceps delivery has also been associated with increased muscle damage and experimentally demonstrated its association with the most common type of pelvic compartment prolapse, the cystocele [116]. The importance of LA activation vs relaxation at vaginal delivery has also been studied. In a study by Parente te al [117], using computational modelling, they demonstrated that maximum pelvic floor straining levels were generated when the head reached station 4 (4cm below the ischial spaines) and that activation of the LA muscle during head descent could generate opposite forces leading to increased pelvic floor injury [118].

The levator hiatus has also been the focus of several pelvic floor imaging studies. Using 2D and 3D ultrasound, Dietz et al established a direct correlation between levator hiatal area and pelvic organ descent [119]. Svabik et al analysed 227 nulliparous women and concluded that the levator hiatus stretch/strain required during childbirth could oscillate between 25% and 245% of the original area [120].

Finally, in a study originally designed to study stress incontinence, it was found that following vagina delivery, only 20% on primiparous women developed a LA injury as seen using MRI. The majority of the damage took plave in the pubovisceral component and only very few occurred in the ile-coccigeal component of the LA. Interestingly, the incidence of LA damage was worse with age and length of the 2nd stage [121]

Fig. 21

Simulated effect of fetal head descent on the levator ani muscles in the second stage of labor. At top left, a left lateral view shows the fetal head (blue) located posteriorly and inferiorly to the pubic symphysis (PS) in front of the sacrum (S). The sequence of five images at left show the fetal head as it descends 1.1, 2.9, 4.7, 7.9, and 9.9 cm below the ischial spines as the head passes along the curve of Carus (indicated by the transparent, light blue, curved tube). The sequence of five images at right are front-left, three-quarter views corresponding to those shown at left. Obstet Gynecol 2004 [112].

Fig. 22

The upper bar graph compares, by muscle, initial and final muscle lengths corresponding to 1.1- and 9.9-cm model fetal head descent, respectively, as shown anatomically in Figure 3. The lower bar graph shows the maximum corresponding stretch ratio found in each levator ani muscle band. Note that the value of the stretch ratio is not simply proportional to initial or final length. For both graphs, muscles are arranged left to right, in ventral to dorsal order of origin location, following the numbering used in Figure 19 [(112)].

1.5 Expression of IGF-1 splice variants in other tissues of the female reproductive system.

The presence of IGF-1 splice variants in muscle following chemical damage has already been documented by Hill & Goldspink [58], when they compared expression of MGF and IGF-1a following mechanical damage by injection of the local anaesthetic bupivacaine into skeletal muscle. During the work conducted for this thesis in muscle, ovarian tissue was used initially as a negative control in our RT-PCR runs. However, the initial sample run showed expression of both IGF-1 splice variants. This finding was persistent in subsequent runs and could reflect the mechanical events taking place within the ovarian epithelium following ovulation, as well as have broader implications of cellular repair mechanisms within human cell pathophysiology.

1.5.1 Expression of IGF-1 splice variants in ovarian tissue

Human ovarian cancer remains the leading cause of death among gynaecologic malignancies in the developing world, and is the fifth most frequent cancer in women [122]. The vast majority of ovarian cancers result from genetic damage accumulated during lifetime which manifests at both chromosomal and molecular levels, and are referred as sporadic cancers. The mutations that lead to the

development of ovarian cancers primarily targets genes involved in regulating proliferation, apoptosis, and senescence [123].

The majority of cancers in the human ovary originate from surface epithelial cells (90%) . Although events leading to ovarian cancer are multifactorial, epithelial cell carcinomas are the only form of ovarian cancer in which progenitor cells are in question [124]. Serous tumours have histological resemblance with the epithelium of the distal fallopian tube (FT), however, the tissue of origin associated with this type of tumour may vary depending on the grade of carcinoma. Two potential sites have been identified as origins for high-grade serous carcinoma: ovarian surface epithelium (OSE)-lined inclusion cysts [125] and the fallopian tube epithelium (FTE) [126]. While the majority of serous carcinomas occur spontaneously, in 10% of cases they are associated with a familial history due to inherited mutations in BRCA1 and BRCA2[127]. Women with BRCA 1 mutation are more likely to have primary FTE carcinomas which resemble histologically serous carcinomas of the ovary. However, even in these genetically predisposed cases, as seen in women with Li-Fraumeni syndrome (exhibiting increased levels of DNA damage in the FTE) [128], inactivation of DNA damage sensing and repair mechanisms seem to play a role in the development of serous cancer with an origin in the FT epithelium [129]. It is likely that

genetic predisposition, anomalies in DNA repair genes and the impact of ovulation [130,131], play a role in cancers originating from the FT and ovarian surface epithelium (OSE). One of the most explored theories alleged to be involved in the genesis of ovarian cancer is the incessant ovulation hypothesis [132], suggesting that repeated ovulations, without long dormant periods, are responsible for transformation of the ovarian epithelium. Ovarian surface epithelial cells overlying the formative ovulatory stroma will show significant genomic damage committing themselves to apoptosis [123]. Contributing factors to this damage include excessive reactive oxidants produced during ovulation and luteal phase, possibly released by leucocytes that infiltrate periovulatory follicles [133], and ischaemia reperfusion flux that accompanies periovulatory tissue remodelling. [134,135,136,137]. Accurate restoration/repair of any potentially harmful mutations would seem essential to prevent further growth of aberrant epithelial cells.

More recently, advances in the understanding of how different paracrine-autocrine growth factors influence ovarian cells behaviour have yield new information on the way a malignant phenotype is facilitated with a distinct growth and metastatic potential. These include the epidermal growth factor (EGF), transforming growth factor (TGF-), platelet derived growth factor, fibroblast growth

factor, vascular growth factor and insulin growth factor (IGF) amongst many others[138].

The aim of this study was to ascertain the expression of the IGF splice variants in human ovarian tissue in the presence of surface epithelial cell damage. Expression of the IGF-1 splice variants may provide more evidence in favour of the incessant ovulation theory as one of the agents involved in the genesis of ovarian pathology. Expression of these repair factors in the presence of OSE damage would suggest that activation of the IGF-1 splice variants would not be limited to stretch forces in muscle only.

1.5.2 Expression of IGF-1 splice variants in myometrium and leiomyomas

Leiomyomas are a major public health problem for women of reproductive age. Uterine leiomyomas, known as fibroids, are benign smooth muscle tumours of the uterus. They represent the most common tumour found in the female reproductive system, seen in 20-25% of all women and are estimated to occur in 40% of menstruating women older than 50 years and are present twice as frequently in black women as in either white or Asian women. They typically resolve after menopause [139].

1.5.2.1 Uterine fibroids classification

1. Submucosal fibroids are the least common. Because they are in the submucosa and near the endometrial cavity, they are associated with heavy and prolonged menstrual periods and an increased miscarriage rate. Submucosal fibroids may be pedunculated and may prolapse through the cervix.
2. Intramural fibroids grow within the uterine wall. Their growth may be associated with mass-related symptoms.
3. Subserosal fibroids develop in the outer portion of the uterus. They may be pedunculated, can potentially grow into the abdomen or in the ligaments of the uterus.

Although most patients are asymptomatic, presenting symptoms most commonly include [(140)]:

- Abnormal uterine bleeding, mainly heavy menstruation called menorrhagia, which is the commonest cause for consulting the doctor.
- Pelvic pain, possibly resulting from intramural degeneration, torsion of a pedunculated fibroid, or intra pelvic pressure
- Abdominal distension
- Infertility

- More rarely, genitourinary dysfunction, which may manifest as increased urinary frequency resulting from bladder compression or flank pain resulting from ureteral compression and hydronephrosis

1.5.2.2. Pathophysiology

Leiomyomas are described as monoclonal tumors derived from a single myometrial cell [141]. However the factors involved in their initiation and further growth remain poorly understood. The neoplastic transformation of the myometrium to leiomyoma is likely to be related to somatic mutations of normal myometrium and complex interactions between sex steroids and local growth factors. However, these somatic mutations are not always present. Other epigenic mechanisms may exist as well. Somatic mutations may include a variety of chromosomal aberrations ranging from point mutations to chromosomal loss of gain. This somatic mutation within the myometrial cell, may be the biologic basis for the differential responsiveness of leiomyomas to a variety of growth promoting factors.

Traditionally, oestrogen has been considered to be the major promoter of leiomyoma growth. Administration of GNRH analogues creating a low oestrogen environment has shown to reduce the size

of leiomyomas. The mitogenic effects of oestrogen are likely to be mediated through other factors and their receptors. There is increasing evidence that oestrogen stimulation of progesterone, epidermal growth factor [142], and insulin growth factor plays a major role in leiomyoma growth [143-5]. Other growth factors identified include the transforming growth factor, basic fibroblast growth factor and vascular endothelial growth factor. The influence of IGF-1 in the pathophysiology of fibroids has already been widely documented [146]. However, whether the two splice variants documented in skeletal muscle, may also be present in the myometrium (smooth muscle) and play a role in the tissue proliferation seen in leiomyomas and in the physiological hypertrophy experienced by the gravid uterus, remains unexplored.

CHAPTER 2

Methods

Ethical approval was obtained from the Royal Free & Medical School Local Research Ethics Committee to recruit and obtain biopsies from the LA muscle in control and the vaginal delivery group, and for the use of archival ovarian and frozen tissue (Appendix 1). All women requiring active sampling were given information sheets about the study and those who agreed to take part were asked to sign a written consent form. A copy of this was then inserted into the patient records.

For the purposes of immunostaining samples were either snap-frozen or placed in formalin and processed routinely through the Department of Histopathology, where the tissue is paraffin wax-embedded.

2.1 Samples

2.1.1 Muscle biopsy technique

Seventeen primigravidae women were successfully biopsied following their first vaginal delivery by the principal investigator. Samples from the pubo-rectalis component of the LA muscle were

obtained vaginally and when the muscle was accessible without further dissection, 1 to 2 cm deep to the right postero-lateral angle of the vagina and 2 to 3 cm above the external anal sphincter (Appendix 2). All women used an epidural as method of analgesia during labour and no other type anesthesia was required to obtain the samples thus avoiding local damage resulting from infiltration of a local anesthetic agent. All samples were obtained within 1 hour of delivery and prior to conventional vaginal reconstructive surgery was performed. Samples were kept in RNAlater stabilization reagent and stored at -80 C.

Samples from the control group were obtained from ten subjects under general anesthetic, using an actuated cutting biopsy needle (Monoject, ABCâ). (Apendix 3). The control group samples were used to compare premenopausal women with women following first spontaneous vaginal delivery in the first study, and to compare differences in expression of the targeted repair factors between pre and postmenopausal women in a second study. The women who agreed to take part were admitted for a diagnostic hysteroscopic and/or laparoscopic procedure for gynecological reasons other than pelvic floor disorders. The pubo-rectalis component was accessible vaginally after digital identification of the LA muscle and the needle

was introduced 2 to 3 cm deep through the right or left postero-lateral angle of the vagina. (Appendix 3). Samples obtained were also kept in RNA later stabilization reagent at -80 C until RNA extraction was later performed. Control samples were obtained from 6 premenopausal (p.102) and 4 postmenopausal (p.108) women. None of the participants in either group had any complications associated with the biopsy sampling procedure either the day of collection or at six weeks follow up.

From an obstetric perspective, one of the limitations of the study is that the premenopausal samples obtained do not reflect the ideal control. In the obstetric scenario, the ideal control case would be the patient opting for an elective caesarean section, i.e, the patient who reaches full term pregnancy uneventfully, without going into labour, and chooses an elective surgical procedure through the abdominal route as the mode of delivery. Although originally this possibility was considered, it was ruled out in the end due to patient safety reasons. As explained above this was the first time this biopsy technique of the LA muscle had been attempted surgically. It involved using a needle cutting biopsy under general anesthetic in a main theatre scenario where resources are available if complications arose. It did not feel appropriate to experiment this technique in an immediately post natal patient in an obstetric

theatre with limited resources and under a regional block. In hindsight, we can now safely document that the procedure itself is practically risk free in the hands of a surgeon with an expertise in pelvic floor anatomy. For the purposes of this study, and from the muscle physiology point of view, our control group fulfilled the criteria required to test our hypothesis as the samples compared reflected the non injured LA muscle under physiological conditions with the over strained LA muscle following vaginal delivery.

2.1.2 Ovarian samples

Samples were obtained from 12 patients who underwent oophorectomey as part of their surgical treatment. Four samples were obtained from post menopausal women diagnosed with epithelial ovarian cancer. Seven samples belonged to premenopausal women whose ovaries showed no pathology on histology and one sample was obtained from a patient diagnosed with ovarian cystoadenoma.

2.1.3 Myometrium and leiomyoma samples

Samples were obtained from three patients. Two patients underwent hysterectomy for pressure symptoms and menorrhagia samples of myometrium and fibroids were taken from these

patients. One patient had a hysterectomy following uterine artery embolisation complicated by fibroid necrosis samples of myometrium and necrotised leiomyoma were obtained from this patient for analysis. Samples were processed using Real Time Quantitative PCR using specific primers for IGF-1 splice variants. Inmuno-staining was performed to confirm the presence of MGF.

2.2 Total RNA isolation

Total RNA was extracted from the muscle samples (mean sample weight 30 mg) using RNeasy Fibrous Tissue kit (Qiagenâ), following the manufacturer s protocol. The frozen weight of the tissue samples was determined prior to homogenization. The extracted RNA was dissolved in RNase-free water and the concentration was determined by spectroscopy at 260nm using the Gene Spec I (Naka Instruments).

2.3 Gel Electrophoresis

PCR products were recovered from the glass capillaries by removing the plastic caps and inverting them into a 1.5ml eppendorf tube, which were then spun down in a bench top centrifuge at no higher than 3000 rpm. 20 l of these PCR products were mixed with 2 l of 6X Loading buffer (Fermentas, UK). The 2%

agarose gel was prepared by adding 2g agarose to 100ml tris/acetate buffer (TAE) and heating in the microwave for approximately 2 minutes to dissolve the agarose. After cooling slightly, ethidium bromide (10mg/ml) was added to the mix, which was then poured into a gel tank and left to set. The PCR products + loading buffer were then carefully loaded into the wells as was a size marker (DNA ladder, Fermentas, UK). The gel was left to run for approximately 45 minutes at 100 volts until good separation of the dye fronts had been achieved. Target specificity was further confirmed by visualizing the product bands under UV light.

2.4 Reverse Transcription

Following RNA extraction, total RNA was then reverse transcribed into cDNA using Omniscript reverse transcriptase (Qiagen, UK). 0.5 g of each sample of total RNA was then mixed with DEPC treated water in a total volume of 10 l and heated to 65 C for 5 minutes before transfer to ice. The samples were then mixed with 2 l First Strand Buffer (10x), 2 l dNTPs (5mM each), 50 pmol random primers or 15 pmol of sequence specific primer, 1 l RNAase inhibitor (10 units/ l) and 1 l Omniscript Reverse Transcriptase (4 units/ l). The reaction volume was made up to 20 l using DEPC treated water. The samples were then incubated

at 37 C for 1 hour followed by 5 minutes at 93 C to inactivate the reverse transcriptase. To facilitate the efficiency of RT of transcripts expressed at low levels such as MGF and IGF-IEa, short specific primers annealing 50-100 base pairs downstream of the polymerase chain reaction (PCR) reverse primers were used. Hence, a mixture of random hexamers and specific decamers were used in the same reaction. The RT primers for MGF and IGF-1Ea are shown in table 1

Primer name	Sequence (5 to 3)	Product size (bp)	Accession no
IGF-1Ea forward	GCCTGCTCACCTTCACCAGC	303	X57025
IGF-1Ea reverse	TCAAATGTACTTCCTTCTGGGTCTTG	-	-
IGF-1Ec (MGF) forward	CGAAGTCTCAGAGAAGGAAAGG	150	U40870
IGF-1Ec (MGF) reverse	ACAGGTAACTCGTGCAGAGC	-	-

Table 2: Table showing reverse transcriptase primers used for MGF and IGF-1ea

2.5 Analysis of Real Time Reverse Transcription-PCR (RT-PCR)

Quantitative Real Time RT-PCR is a highly sensitive procedure that allows quantification of transcripts in low abundance. It s for this reason that a Light Cycler machine (Roche Diagnostics, UK) was used in the present study. Incorporation of SYBR Green I dye

during real-time PCR permits the detection and quantification of newly synthesized DNA, as well as verification of product authenticity with the in-built melting curve analysis procedure.

SYBR Green I dye binds to the minor groove of double-stranded (ds) DNA and therefore different intensities of fluorescence signals can be detected during the various stages of PCR depending on the amount of ds-DNA present. During denaturation of ds-DNA to single-stranded DNA, SYBR Green I is released. As described by Hameed *et al* [(93)], a standard (Fig. 23) curve was created from dilution series of standard DNA (which contained the target sequence of interest) of known concentrations was included in each run, and it was relative to this that samples of unknown concentration were quantified. A negative control was present in each run in which the template DNA was replaced with RNase-free water. Target specificity was further confirmed by running samples on an agarose gel.

Upon annealing, PCR specific primers hybridise to the target sequence resulting in small parts of ds-DNA to which SYBR Green I dye binds and fluorescent intensity increases. Finally, in the elongation phase, when the PCR primers are extended, the entire DNA target becomes double-stranded, and a maximum amount of dye is bound. Fluorescence are recorded at the end of the

elongation phase and increasing amounts of PCR products are monitored form cycle to cycle and visualised in real-time as they occur.

Nevertheless, this technique is not exempt of possible signalling errors. For example, in the particular case of a signal being generated by SYBR Green I binding to double stranded by-products such as primer-dimers, which result from non-specific annealing and primer elongation events. These events occur as soon as PCR reagents are combined. The formation of primer-dimers competes with formation of PCR product, leading to reduced amplification efficiency and a less specific PCR test. In order to counteract this situation, a Hot Start method was used where an anti-Taq DNA polymerase antibody was included and added to the reaction mixture to inactivate the enzyme until the temperature reached about 70 C. A typical experimental protocol using the appropriate primers (Table 2) contained four programmes and the levels of specific mRNA were determined per g of total RNA. All the PCR primers were designed using Omega Version 2.0 (Oxford Molecular) and synthesised by Sigma Genosys.

Each dsDNA product has its own specific melting temperature (TM), which is defined as the temperature at which 50% of the DNA

becomes single-stranded and the rest remains double-stranded. This is determined by the length and G+C content of the fragment. Sequence confirmation of the amplified product, once the amplification cycles are completed, is achieved by melting curve analysis (Fig 24). During this analysis, products are denatured at 95 C, annealed at a specific temperature (usually 5-10 degrees higher than that of the PCR annealing temperature) and then slowly heated to 95 C where fluorescence is measured at every 0.2 C increase. Using a positive standard as a control during the PCR run, melting temperatures of the unknown samples were compared with that of the control, to separate the target confirmed by the PCR signal form those of non-specific products (signals generated by the by-products have a TM lower than that of the specific product). This way, small amounts of sequences of interest could be identified through the melting curve analysis.

Fig. 23: Graph showing standards reference values used as controls during PCR run

Fig. 24

A - Example of the screen the user is presented with once the run is complete

B - Graphs showing the melting curve profile of the specific products formed during the PCR

2.6 Immuno-staining

In order to verify expression of the targeted proteins in the analyzed tissues Immunostaining was conducted on some of the samples studied. The tissues stained included :

1. Premenopausal control LA muscle
2. LA muscle samples following spontaneous vaginal delivery
3. Normal ovary
4. Ovary diagnosed of epithelial ovarian cancer
5. Myometrium
6. Leiomyoma

2.6.1 Paraffin sections

Samples were fixed in formalin and paraffin-wax embedded. A prerequisite for all histological and cytological investigations is to ensure preservation of tissue architecture and cell morphology by adequate and appropriate fixation. The demonstration of many antigens depends heavily on the fixative employed and the immunocytochemical method selected. There is no one fixative that is ideal for the demonstration of all antigens. However, in general, many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded material. The most widely used fixatives in diagnostic hospital histology laboratories are formalin based. In this study, either 10% formal saline or 10% neutral buffered saline (10% w/v formaldehyde in water) was used. Formalin is a neutral salt employed to maintain tonicity. The fixative frequently contains a buffering system to maintain the pH. This is well tolerated by

tissues and has a good penetration though it is important to note that in large specimens, fixation may extend beyond optimal times and may cause shrinkage or distortion. Tissues were processed using an enclosed automatic processing system (VIP 2000F/300E) programmed with the following schedule: 10% neutral buffered formalin two hours at 40 C, 70% industrial methylated spirit (IMS) one hour at 40 C, 90% IMS one hour at 40 C, absolute IMS three hours at 40 C, xylene four hours at 40 C, paraffin wax for three hours at 60 C. Tissues were embedded in paraffin wax utilising Tissue-Tek III.

2.6.2 Frozen sections

Although there are an increasing number of antibodies available that will demonstrate cell membrane-associated antigens in paraffin sections, the use of frozen sections still remain essential for demonstrating many antigens. Frozen sections have certain inherent disadvantages when compared to their corresponding paraffin counterpart and are technically difficult to cut. All frozen tissues were stored at -80 C. Prior to storage, the tissue was placed on cork using Cryo-M-Bed; the cork and tissue were submerged into liquid nitrogen to ensure adherence. Sections were picked up on 3 aminopropyltriethoxysilane (APES) coated slides

and air-dried for approximately fifteen minutes. The slides were then wrapped in cling film and stored at -20 C to prevent water loss.

2.6.3 Antibodies

Antibodies are produced in vertebrates as a defence against infection. They are made by a class of white blood cells, called B lymphocytes or B cells. Each antibody molecule is made up of two identical light chains and two identical heavy chains, so the two antigen binding sites are identical.

Briefly, antibodies can be made in the laboratory by injecting an animal (usually a mouse, rabbit, sheep or goat) with an antigen; for example, antigen A. Repeated injections of the same antigen at intervals of weeks stimulates specific B cells to secrete large amounts of anti-A antibodies into the blood stream. As many different B cells are stimulated by antigen A, the blood will contain a variety of anti-A antibodies, each of which binds A in a slightly different way. There are two categories of antibodies; polyclonal and monoclonal antibodies. Polyclonal antibodies are a mixture of a family of antibodies to different epitopes in the antigen. Monoclonal antibodies are directed against a single epitope, hence are usually more specific than polyclonal antibodies and serve as powerful

tools in the investigation of macromolecules within cells [147]. Monoclonal antibodies are inherently much purer reagents than polyclonal antibodies and display the most desirable attributes (high affinity and exquisite selectivity) [148]. The main advantage of monoclonal antibody over polyclonal antibodies is the possibility of background-free staining that result from insignificant contamination by other irrelevant antibodies. However, the major disadvantage of monoclonal antibody is that their affinity for specific antigens is usually less than the affinity of polyclonal antibodies of similar specificity [148]. Polyclonal antibodies are produced by different cells and therefore can react with various epitopes on the antigen against which they are raised. In the present study, staining was conducted using a polyclonal antibody for the unique C terminal (E domain) of MGF.

2.6.4 Standard IHC Protocol

10 m size sections were prepared from these samples. Sections were deparaffinised in xylene and rehydrated in serial dilutions of methanol. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 minutes, sections were then blocked in a 10% solution of goat serum (Sigma) in PBS for 60 minutes at room temperature. The sections were then

incubated with polyclonal anti-MGF antibody (raised in rabbits, diluting at 1:400) in PBS at 4 C overnight. A negative control was carried out by substituting the primary anti-MGF with PBS buffer. Secondary Biotinylated goat anti-rabbit IgG (Dako , UK) was then added for 30 minutes, followed with peroxidase-conjugated streptavidin-biotin (Dako) at a dilution of 1:200 for 30 minutes. The immunocomplex was visualized by incubating the sections in a solution of 3,3 -diaminobenzidine (Sigma , Aldrich, UK) in PBS. Sections were finally dehydrated in serial dilution of methanol and xylene, and mounted in DPX. The specificity of the anti-MGF antibody was characterized by neutralizing the antibody using MGF peptide or substituting the primary antibody in the immunostaining procedure (Table 2). Slides were visualized under light microscope with a magnification of 200 x.

Antigen	Antigen retrieval	Primary antibody dilution	Incubation time for primary antibody	Incubation temperature for primary antibody
MGF	None	1:70	overnight	4 C

Table 3

2.7 Updated literature review

Following the oral viva on the 27th of July 2011, it was agreed by the examiners that it would in the best interest of the work submitted to conduct a systematic review for the period 2007-2011. The following section describes the methodology undertaken to conduct the updated literature review:

Medline / Embase search strategy

1. IGF-1
2. Insulin-like Growth factor 1/I
3. IGF-1 splice variants
4. IGF-1Ec
5. IGF-1Ea
6. Levator ani muscle
7. Levator ani injury
8. Pelvic floor muscle
9. Pelvic floor injury
10. Mechano Growth Factor
11. Vaginal delivery
12. Obstetric injury
13. Spontaneous vaginal delivery

14. Second stage of labour

Results of the search history are shown in Appendix 4.

Search history included 186 electronic searches combining the terms shown above. Electronic searches were also conducted in the Cochrane database. Searches were conducted monthly until the date of resubmission. Most of the literature available to date on the assessment of the levator ani muscle injury associated with vaginal delivery has been produced within the field of imaging and updated references have been added to the main text. The field of skeletal muscle injury in correlation with damage markers and related pathophysiology has produced fewer articles which have also been incorporated into the main text. Literature search updates were also conducted in the aetiology of ovarian cancer. When searches combined the specific muscle damage markers and different obstetric search variables, the only article published to date since 2005 remains the work described in this thesis (Appendix 4). In total 31 references were added to the original text submitted. The majority of these articles reflect the scientific progress experienced on the proteins studied over the past few years, however they did not alter the scientific value of the work

presented, although they have added complexity to the understanding of tissue damage at cellular level.

CHAPTER 3

Results

3.1 IGF-1 splice variants expression between control premenopausal group and the vaginal delivery group.

The length of the first stage of labour in the delivery group ranged between 4h 30 min to 10h (mean 7h). The length of the second stage described as time between full dilatation and delivery of the baby ranged from 1h 20 min to 3 h (mean 2h 12min). There was one case of precipitate labour where the second stage was recorded as 10 minutes. The weight of the babies at delivery ranged between 2830g and 4114g (mean 3421g). All the cases were sutured by the principal investigator. One patient suffered a perineal haematoma from multiple tears not related to the site of the biopsy.

Haemostasis following biopsy extraction in the pre and post menopausal group was achieved by one or two interrupted absorbable sutures. There were no post-operative complications in any of the cases sampled using the cutting needle biopsy as described in the methods chapter.

3.1.1 RT-PCR results

RNA extraction was performed with concentrations ranging from 0.05 to 0.14 g/ l . Out of the 17 samples initially biopsied, 6 of them had insufficient RNA extraction (concentration <0.05) and 1 sample was damaged during the process of tissue homogenization using the rotor homogenizer. MGF and IGF-1Ea mRNA levels are represented in tables 4 and 5 (Fig. 25, 26, 27) . Thirteen samples were biopsied on premenopausal participants. Out of this 13, five samples produced RNA concentrations of less than 0.05 and 2 were lost during the process of mechanical homonization. The mean value for MGF in the control premenopausal group was $2.35x10^{-8}$ g/ l and $2.52x10^{-6}$ g/ l in the delivery group. Likewise, IGF-1Ea mean value for the premenopausal group was $6.02x10^{-6}$ g/ l and $1.0 \ x10^{-3}$ g/ l for the study population. MGF and IGF -1Ea mRNA levels were significantly increased (100 and 1000 fold respectively) in the delivery group as compared to the control population, p = 0.012 and p = 0.04 respectively. Analysis of the different variables in the delivery population, suggest there was a positive correlation between length of the second stage and MGF and IGF-1Ea expression (r = 0.56 and p = 0.08; r = 0.43 and p = 0.2 respectively). There was no correlation between the length of first stage, weight of the babies or women s age.

N	Age	Maternal Height (cm)	Maternal Weight (Kg)	Parity	Years post delivery	MGF (g/ l RNA)	IGF-1Ea (g/ l RNA)
1	21	153	88	0	0 (nulliparous)	$1.52x10^{-08}$	$1.23x10^{-06}$
2	26	158	59	0	0 (nulliparous)	$1.50x10^{-09}$	$3.14x10^{-07}$
3	46	162	73	2	12 (C/S* x2)	$6.29x10^{-08}$	$2.42x10^{-06}$
4	32	160	62	0	0 (nulliparous)	$2.34x10^{-08}$	$1.48x10^{-06}$
5	27	168	75	0	0 (nulliparous)	$3.03x10^{-07}$	$4.38x10^{-04}$
6	45	156	62	2	15	$1.48x10^{-08}$	$1.00x10^{-09}$

*C/S: caesarean section

Table 4: IGF-1 splice variants expression in pre-menopausal control group

N	Age	Baby s Weight	1st stage*(h)	2nd stage**(min)	Episiotomy/ tear	MGF (g/ l RNA)	IGF-1Ea (g/ l RNA)
1	30	3516g	6	80	Tear	$3.81x10^{-08}$	$3.55x10^{-05}$
2	34	3044g	6	120	Tear	$2.28x10^{-08}$	$1.16x10^{-07}$
3	26	3494g	12	180	Tear	$2.38x10^{-06}$	$9.74x10^{-04}$
4	26	2830g	4	180	Tear	$2.14x10^{-06}$	$1.27x10^{-03}$
5	37	3034g	8	167	Tear	$3.41x10^{-06}$	$2.10x10^{-03}$
6	26	3510g	1	10	Tear	$1.81x10^{-08}$	$1.34x10^{-03}$
7	18	4114g	10	180	Tear	$3.35x10^{-06}$	$1.11x10^{-03}$
8	33	3826g	8	143	Tear	$1.53x10^{-07}$	$3.76x10^{-05}$
9	29	3126g	9	100	Episiotomy	$4.93x10^{-06}$	$4.05x10^{-08}$
10	34	3300g	7	180	Tear	$8.76x10^{-06}$	$3.16x10^{-03}$

* hours; ** minutes.

Table 5: IGF-1 splice variants expression in women following vaginal delivery.

Study Group

Fig. 25
In the delivery population, there was a positive correlation between length of the second stage and MGF and IGF-1Ea m RNA expression (r = 0.56 and p = 0.08; r = 0.43 and p = 0.2 respectively)

Fig. 26
MGF and IGF-1Ea mRNA expression in the control population (g/ l RNA)

Fig. 27
Exponential representation of MGF and IGF-1Ea mRNA levels increase in the delivery group as compared to the control population, p = 0.012 and p = 0.04 respectively.

3.1.2 Immunostaining results from the control and vaginal delivery group using polyclonal anti-MGF antibody.

Fig. 28A . Premenopausal LA muscle

Fig. 28B. Post partum LA muscle

Fig. 28C. Post partum LA muscle

Fig. 28D. Blocked antibody sample

Fig. 28.
LA muscle sample stained with anti-MGF antibody from a control sample (27A) and a study group sample (27B). The control sample appears mildly stained and conserves its muscle architecture. The sample from the study group shows histomorphological changes of eccentric damage and appears highly stained when compared with the control group.
Magnification (200x). In order to demonstrate specificity of the MGF antibody, a different LA muscle sample from the study group, (27C), was tested by blocking the antibody with the MGF peptide (27D). Magnification (200x).

3.2 LA IGF-1 splice variants expression in the control group and the menopausal group. RT-PCR results

Fifteen post-menopausal women underwent LA biopsy in theatre. Out of this 15, four were lost in the process of homogenization and 7 did not reach satisfactory RNA concentrations. Premenopausal women expressed both MGF and IGF-1a splice variants with only sample 2 no detection of MGF and sample 6 not expressing IGF-1a. MGF was not detected in all postmenopausal samples, whilst IGF-1a was present in all samples. (Table 7)

Premenopausal	Age	MGF(g/ l RNA)	IGF-1a(g/ l RNA)
1	21	$1.52x10^{-08}$	$1.23x10^{-06}$
2	26	$1.50x10^{-09}$	$3.14x10^{-07}$
3	46	$6.29x10^{-08}$	$2.42x10^{-05}$
4	32	$2.34x10^{-08}$	$1.48x10^{-06}$
5	27	$3.03x10^{-07}$	$4.38x10^{-04}$
6	45	$1.48x20^{-08}$	$1.00x10^{-09*}$
Postmenopausal		MGF	IGF-1a
7	51	**$1.50x10^{-09}$**	$4.14x10^{-07}$
8	61	**$1.50x10^{-09}$**	$2.56x10^{-07}$
9	53	**$1.50x10^{-09}$**	$4.33x10^{-07}$
10	65	**$1.50x10^{-09}$**	$1.03x10^{-04}$

Table 7. Expression of IGF-1 splice variants in pre and post menopausal women.

* 10^{-09} shows no detection of targeted protein. Parity in the post menopausal women ranged between P1 and P3

3.3 Variations inherent in the sampling procedure.

Although a total of 45 patients were initially recruited for the study, only 20 samples produced sufficient RNA concentrations to complete the RT-PCR analysis. Several factors could have contributed to this including the nature of the blind technique used to biopsy the LA muscle (digital palpation) as well as the anatomical

and physiological changes experienced by the muscle during trauma at the time of delivery or aging associated changes (sarcopaenia/atrophy) seen in the post menopausal women. This is more relevant in the control and post menopausal samples as the cutting needle biopsy was only 1 mm diameter. Samples from the delivery population were obtained at the time obstetric injury under conditions were the need for prompt haemostasis may have conditioned the accuracy of the sample obtained facilitating contamination by other tissues such as fat or connective tissue.

Since this study was conducted, there have been significant advances in pelvic floor imaging. The use of new imaging facilities at the time of LA muscle biopsy in the future, will contribute to a more precise sampling technique.

3.4 IGF-1 splice variants expression in normal ovary and ovarian cancer.

Both IGF splice variants were highly expressed in both control and pathological samples (Table 8 and 9). Concentrations seen in the control group are similar to those seen in previous studies performed in muscle tissue. Although our numbers are small for the usual statistical analysis, when comparing both groups there is a

trend in the pathological group to show higher concentrations of both IGF-1a and MGF when compared to the control group. In order to enable any interpretation of this finding, further studies are needed using larger sample sizes.

N	Ovarian histopathololology	MGF (g/ l RNA)	IGF-1a (g/ l RNA)
1	Ovarian Cystoadenoma	2.79x10-08	1.14x10-06
2	Normal ovary	4.32x10-08	1.66x10-06
3	Normal ovary	1.27x10-09	2.44x10-06
4	Normal ovary	2.55x10-08	1.05x10-06
5	Normal ovary	7.62x10-09	1.67x10-06
6	Normal ovary	1.36x10-09	1.69x10-06
7	Normal ovary	1.66x10-08	1.50x10-06
8	Normal ovary	1.69x10-09	1.86x10-06

Table 8. IGF-1 splice variants in patients with benign ovarian histology

N	Ovarian histopathololology	MGF (g/ l RNA)	IGF-1a (g/ l RNA)
1	Ovarian CA	1.23x10-07	2.42x10-04
2	Ovarian CA	1.50x10-09	3.70x10-04
3	Ovarian CA	2.25x10-07	6.35x10-04
4	Ovarian CA	6.82x10-07	7.53x10-09

Table 9. IGF-1 splice variants in patients with ovarian cancer

3.4.1 Immunostaining in normal ovary and ovarian cancer with polyclonal MGF antibody

Immunostaining of the normal and pathological ovaries confirms the presence of MGF in both samples (brown). Repair activity, is seen in the normal ovary following physiological follicular activity (Figure 28A). However, staining in the ovarian cancer sample (Figure 28B) surrounding the tumour area appears significantly increased consistent with the increased MGF levels seen in RT-PCR concentrations in these samples.

Fig 29A Fig 29B

Fig. 29

Normal ovarian tissue (29A) showing repair activity (MGF expression) surrounding follicular activity. In the ovarian cancer tissue (29B) there is increased repair activity around and within the tumour area.

3.5 Expression of IGF-1 splice variants in myometrium and leiomyoma. RT-PCR results.

All the samples analyzed expressed both MGF and IGF-1a in both myometrium and leiomyoma, except for the embolised sample

which due to necrosis was unsuitable for further analysis (Table 10). While the samples obtained from the two patients undergoing a total abdominal hysterectomy (patients 1 and 2) expressed similar MGF levels to the levels seen in the myometrium sample from the embolised uterus (patient 3), the IGF-1a concentration seen in the embolised myometrium was markedly reduced when compared with the levels seen in the myometrium of patients undergoing routine abdominal hysterectomy in a normally perfused uterus.

Participants	Specimen	MGF (g/ l RNA)	IGF-1a (g/ l RNA)
Patient 1	Myometrium	4.51x10-06	8.42x10-04
	Fibroid (10cm)	1.38x10-06	2.94x10-04
Patient 2	Myometrium	2.41x10-06	9.97x10-04
	Fibroid (11cm)	3.54x10-06	2.05x10-03
Patient 3 (embolization)	Myometrium	4.41x10-06	**3.73x10-08**

Table 10.
Expression of IGF splice variants in myometrium and leiomyoma. IGF-1a levels in patient 3 (bold) are significantly lower than levels seen in normally perfused myometrium and fibroid (patient 1 & 2).

3.5.1 Immunostaining of myometrium and myoma using polyclonal MGF antibody.

Immunostaining was performed in three different samples:

- the normally perfused myometrium (Fig. 30A)
- leiomyoma (Fig. 30B)
- embolised myometrium following UAE (Fig. 30C).

Samples from the necrotised leiomyoma were not suitable for immunoanalysis. MGF staining was more pronounced on the

leiomyoma sample (Fig. 30B) compared with the normally perfused myometrium (Fig. 30A) and the UAE sample (Fig. 30C). The myometrium sample following UAE also stained to MGF although expression was less homogeneous that in normal myiometrium (Fig. 30A).

Fig. 30

Immuno staining of normal myometrium, leyomyoma and embolised myometrium.

Fig. 30A. Normally perfused myometrium

Fig. 30B. Leiomyoma

Fig. 30C. Myometrium following uterine artery embolisation

CHAPTER 4:

Discussion

4.1 Expression of IGF-1 splice variants following vaginal delivery

LA muscle damage following vaginal delivery has already been widely described using different imaging techniques. Clinically, evidence of muscle damage following parturition, is widely accepted and several studies have identified the puborectalis as the LA muscle component most affected [149,150]. In the present work, RT-PCR analysis of samples showed a marked up-regulation of MGF (human IGF-1Ec) and IGF-1Ea in the pubo-visceral component of the LA muscle, following vaginal delivery. It has been previously shown that following a bout of muscle damaging eccentric cycling exercise in adults [55], the levels of MGF were also significantly up-regulated. As seen in the LA stretch model mimicking a vaginal delivery devised by Lien et al [112], the maximum pubococcygeal muscle stretch ratio of 3.26 found in this study exceeds by 217% the largest non-injurious stretch (1.5 stretch ratio, upper limit of shaded region, see fig. 20) observed in nongravida passive striated appendicular muscle. This supports the findings of the present study where the levels of MGF and indeed

IGF-1Ea detected in the post-parturition group reflect this severe mechanical challenge following labour and vaginal delivery.

Using a rat model, Hill and Goldspink [58] studied MGF and IGF-1Ea expression following mechanically and chemically induced muscle damage, and found that MGF expression preceded IGF-1Ea expression after stretch and overload. Assessment at frequent intervals revealed that MGF expression reached a peak within one day following mechanical damage. On the other hand, IGF-1Ea was expressed later, reaching a peak level on day 11 whilst at the same time MGF expression progressively decreased as the splicing of the IGF-1 gene to MGF indicated that MGF was more of the nature of a pulse of a day or so. This lack of correlation in the pharmacokinetics between MGF and IGF-1Ea expression was also noted by Hameed *et al* [93] following weight lifting exercise. Evidence that these two splice variants of IGF-1 have not only differential expression but have different functions, has been demonstrated by recent *in vitro* experiments by Yang and Goldspink [62] and Hill and Goldspink [58]. MGF during the first phase of this repair process caused rapid proliferation of mononucleated myoblasts with no further differentiation into myotubes. IGF-1Ea enhanced terminal differentiation and fusion of the satellite cells

with the damaged muscle fibres which, with the general up-regulation of protein synthesis, are an essential part of the second phase of repair and remodelling of muscle.

As discussed previously on the methodology chapter, it was not possible for ethical reasons to ascertain MGF and IGF-1Ea pharmacokinetics throughout pregnancy and whether an increased intra-abdominal pressure of the gravid uterus may pose a gradual physiological stress to the LA muscle during pregnancy therefore triggering to a certain extent, the splicing of the IGF-1 gene. However, the markedly up regulated MGF levels seen within one hour of vaginal delivery when compared to the control group in this study, and Hameed *et al* [93] results on their study group (after high resistance exercise), suggest that this could be a direct response to muscle damage as described by Hill and Goldspink [58] following acute stretching forces occurring during the second stage of labour (eccentric damage). This would be supported by the positive correlation seen between length of the second stage, the most relevant insult to the muscle during labour, and the expression of IGF-1 splice variants ($r = 0.56$ and $p = 0.08$; $r = 0.43$ and $p = 0.2$ for MGF and IGF-1a respectively). On the other hand, the previously documented time gap of 24 to 72 hours seen in between the

expression of these two peptides [63,66,67] and the finding of such high levels of both isoforms in one single sampling exercise 1 hour post delivery, would suggest that this repair mechanism would have been activated prior to the actual second stage of labour or may be during labour itself.

Analysis of individuals, revealed two subjects (numbers 6 and 9, Table 4 in the results chapter) that differed in their response between MGF and IGF-1Ea expression; subject 6 showed high levels of IGF-1Ea but relatively low levels of MGF, with the reverse findings seen in subject 9. This may be due to differences in timing and expression kinetics of these growth/repair factors. Subject 6 in particular, who had a precipitate labour and a second stage of approximately only 10 minutes, expressed one of the lowest MGF concentrations. One explanation for this difference would be insufficient time for the IGF-1 gene to be up-regulated and spliced to MGF (1hour first stage + 10 minutes second stage), and hence promote the repair cascade. If this is the case, it would suggest a more acute muscle response to labour itself rather than pregnancy as a whole when compared with those deliveries that took between one and three hours of second stage. The high levels of IGF-1Ea seen (similar to the other groups) in combination with low levels of MGF may reflect a different pathway for IGF-1Ea which is not

restricted to its local injury input. IGF-1Ea may reach the damaged areas systemically as well, as it is suggested later when discussing tissue damage in smooth muscle. This presumably is supported by the findings on subject 9 where an episiotomy was performed. Episiotomy is a routine obstetric procedure where the perineum is incised laterally through the vagina to allow the delivery of the head, in cases where there is delay in foetal progress or compromise is suspected during the second stage of delivery. In many occasions the incision extends beyond the LA muscle and as IGF-1Ea is partly delivered systemically, this deficit in blood perfusion could explain the low levels of this protein seen in this particular patient, the only one who had an episiotomy. However, more recently, the sole role attributed to IGF-1Ea as main contributor to the second step of muscle proliferation has been questioned. In a study by Matheny RW et al [(151)], it has been suggested that the myoblast differentiation process could be up-regulated by IGF-1 splice variants through the actions of mature IGF-1 and not only IGF-1Ea. Although this finding does not question IGF-1Ea attributes in the muscle differentiation process, it does blur the boundaries between these two factors involved in muscle repair.

These findings cannot *per se* establish whether these differences are related to pregnancy itself, vaginal delivery, or a combination of both. However, they show that this muscle repair mechanism is detectable and reflects the level of mechanical stress that the LA muscle is undergoing during pregnancy and vaginal delivery.

Damage inflicted to the LA muscle (active support) and attached fascias (passive support) may have short and long term implications in women s pelvic floor function including both its support (prolapse of the three compartments of the vagina) and continence mechanisms (stress urinary incontinence and/or anal incontinence).

Before firm conclusions could be drawn about the expression of these repair factors following vaginal delivery and long term pelvic floor performance, further studies are required with larger number of patients in which variables such as length of first and second stage of labour, ethnicity, parity, age differences, collagen type variations and mode of delivery (forceps, suction ventouse, tear or episiotomy, and elective caesarean section), are also analyzed.

Although the present repair mechanism seems to act upon the muscle which has been damaged, published imaging studies using 3D MRI on the LA muscle months after vaginal delivery have shown that physical tearing of the LA muscle occurring during

delivery (macro damage) leading to complete dissection of the muscle remains disrupted long after [152]. This would suggest that although this repair mechanism may facilitate repair at a cellular level (micro damage), there may be a limitation for this mechanism when the muscle is physically torn apart.

Finally, it was discussed in the introduction the role of MGF as a neuroprotective agent. Whether this has short or long implications in pelvic floor recovery following vaginal delivery remains unexplored. It has been demonstrated that 1 in 4 women will experience neuropathic injury to the LA muscle following vaginal delivery as seen using electromyography [153]. There is also recent evidence that approximately 30% of those women who may have experienced neurological damage to the LA at delivery, will recover by 6 months post partum [154]. To what extent MGF expression may contribute to this reinnervation process, remains an area for future research.

4.2. Premenopausal and post menopausal expression of IGF-1 splice variants

Expression of MGF and IGF-1Ea by the LA muscle in a control premenopausal population was also seen where the muscle was not submitted to the same physiological levels of mechanical strain.

The six pre-menopausal samples obtained belonged to continent women. Four of them had never been pregnant, one participant had her last delivery 12 years ago having had two elective caesarean sections without going into labour, and one woman had two uncomplicated vaginal deliveries, with the most recent one having taken place 15 years ago. Expression of IGF-1 splice variants in this group could reflect the physiological response (associated resting electrical activity) that LA muscle exerts to counteract changes in intra-abdominal pressure that take place during daily activities as well as a consequence of its daily physiological activity to preserve both urinary and anal continence. Expression of IGF-1 splice variants in the LA muscle in the absence of overstretching, suggest that daily continence mechanism poses a level of strain to the LA muscle which requires a continuous muscle repair capacity to regenerate its fibres. Comparing the results on the control group with the delivery group, it becomes evident the significant difference in expression of the splice variants between both groups. This would be expected given the difference in muscle overload. MGF and IGF-1Ea mRNA levels were significantly increased (100 and 1000 fold respectively) in the delivery group when compared to the control pre-menopausal population, $p = 0.012$ and $p = 0.04$ respectively.

4.3. Expression of IGF-1 splice variants in the postmenopausal population.

The process of sarcopenia described as the natural loss of muscle mass and loss of muscle strength associated with ageing, have been widely discussed in the literature. Whilst the pre-menopausal group in the present study showed expression of MGF in all its participants except in one, the post menopausal group failed to express this splice variant in all the participants. This failure to express MGF in the elderly group, may explain two main clinical facts in elderly female patients. Firstly it may help to understand why most female patients suffering from incontinence show symptoms later in life. Failure to express MGF may lead to impaired regeneration of muscle which will translate in a less effective tool to provide its support and continence function. A second area in which failure to express MGF may have an impact in elderly women compared to younger women, is on those patients undertaking pelvic floor muscle exercises as a conservative treatment for urinary stress incontinence.

Pelvic floor muscle (PFM) exercises are the first step in the management of urinary incontinence related to straining or exercise, known as stress urinary incontinence (SUI), -prevalence estimated between 4-35%- [155]. Their validity as a first line

treatment option has been recently confirmed by two Cochrane database systematic reviews [156,157]. They are routinely successfully implemented in the antenatal and post natal period however, successful rates treating SUI using different protocols have been reported only at about 60%. Their efficacy has also been demonstrated in patients with other urinary conditions such as urgency and urinary urgency incontinence [158]. Sampselle et al. [159] and Fantl et al. [160] recommended 30 to 45 contractions a day. Contractions should be sustained for 6 to 8 seconds to activate both slow and fast-twitch muscle fibres. A 10-second contraction activates slow-twitch fibres first and quickly activates the more fatigable fast-twitch fibres. The activation of slow-twitch fibres probably accounts for the sustained effort. Since the strongest stimulation for strength increase is the intensity of the contraction, the object of training is to recruit as many motor units as possible including both fast twitch and slow-twitch fibres [161-2]. PFM exercises can be taught to patients individually using techniques outlined by Sampselle and colleagues [159].

Recommended protocols for PFM exercise training vary between 4 and 20 weeks, but Domounly et al [163] demonstrated the greatest improvement in PFM strength and in urine loss in the first 8 weeks of a 16-week protocol. In a recent review by the Cochrane library it

is acknowledged the wide variation in PFM training regimes available, however it provided further support to the widespread use of PFM training as the first line conservative treatment for women with SI and mixed incontinence [157].

Poor expression of MGF in the LA of postmenopausal women, as expected from studies conducted in the elderly population [164], would lead to a decreased capability of the LA muscle to recruit new muscle fibres, therefore conditioning the efficacy of the PFM exercises. This finding would support the clinical evidence suggesting that pelvic floor exercises are a less successful therapeutic option in elderly patients when compared with younger women [165-6]. However, other factors play a role in PFM exercise adherence by patients, also relevant for therapy success. Evidence has shown that for a successful PFM exercise program, basic understanding about the purpose of the muscle training, the anatomy of the pelvic floor, and the characteristics of effective and ineffective contractions need to be taught. Some or all of these aspects may be more difficult to assimilate by the elderly population. Further research on MGF expression and the differences seen on PFM exercises between pre and postmenopausal women, may help to clarify this.

Another interesting finding in the postmenopausal samples is that the levels of expression of IGF-1Ea are not significantly lower in the elderly group compared with levels seen in premenopausal women. This would suggest a different pathway for the splicing of IGF-1Ea, not restricted only to the actual injury mechanism. However, since IGF-1Ea requires de novo myoblast cells provided by MGF to form new muscle, in the absence of the latter, the regenerating process of new muscle would be hampered.

Finally, in order to demonstrate the presence of the targeted proteins by the LA muscle we used immunostaining to compare expression between the premenopausal and the delivery group. Structural damage to skeletal muscle following eccentric exercise, i.e., when muscle is lengthened leading to no overlap between the actin and myosin filaments, has been described as sarcomere-length inhomogeneities or the popping sarcomere hypothesis [(85)]. In the delivery group (Fig. 23B), when overstretched muscle fails to re-interdigitate following relaxation after overstraining, disruption changes in fibres patterns, may lead ultimately to non-functional sarcomeres and fibre death. This damage in the muscle fibre would be consistent with the highly stained muscle sample seen in the study. On the other hand, samples from the control group, which

maintained the normal muscle architecture, although appeared positively stained to MGF the intensity seen on the slide was less when compared with the delivery group, corroborating that MGF expression is increased following muscle overload.

4.4. Expression of IGF-1 splice variants in other gynaecological organs in the presence of damage not associated with overload.

Most of the research presented in this thesis describes the expression IGF-1 splice variants following skeletal muscle injury. However, during the search for a negative control in order to run the muscle samples using RT-PCR, ovarian tissue was processed expecting negative expression of the targeted proteins. To my surprise, this was not the case. Encouraged by this finding it was hypothesized that these repair factors should also be expressed in smooth muscle tissue due to its close structural similarities with skeletal muscle. The remaining part of this thesis explores the expression of IGF-1 splice variants in the human ovary and myometrium. Confirmation of their expression both under physiological and pathological conditions opens new venues for research in the future.

4.4.1. Expression of IGF-1 splice variants in normal and pathological ovarian tissue.

Several hypothesis have tried to explain over the past few decades the sporadic natural history of ovarian cancer; the incessant ovulation hypothesis [132], the pituitary gonadotrophin hypothesis [167], the androgen/progesterone hypothesis [168] and inherited mutations of the BRCA1 and BRCA2 genes [127] . Although all of them contribute to explain possible mechanisms for the development of the disease, there is not as yet, a unifying hypothesis to account for the sporadic cases of ovarian cancer. The expression of IGF-1 splice variants described in this work would initially support the hypothesis behind the incessant ovulation theory, which postulates that tumours result due to the recurrent trauma to epithelial ovarian cells as a result of ovulation. Each ovulatory cycle would cause the ovarian surface epithelial layer to undergo proliferation and repair. The more times the cells have to undergo repair, the greater the chance for errors to occur during the repair process. Expression of both IGF splice variants in ovarian tissue would corroborate that this process takes place in the ovary both during physiological ovulation and in ovarian pathology. However, several aspects of this process remain unanswered. This

repair mechanism seen through MGF and IGF-1Ea does not appear to be restricted to ovarian tissue only and it would appear to be more a standard physiological response triggered by damaged tissues to achieve repair. Although initially the mechanism triggering splicing of the IGF-1 gene was thought to be an autocrine response to mechanical injury, later studies by Hill and Goldspink [58] found that the same response was obtained following chemical damage with bupivacaine, a commonly used local anaesthetic. Which chemical or environmental agents promote splicing of the IGF gene remains unknown, as well as whether these may be different depending on the tissues analyzed. The findings in the ovary would support Hill and Goldspink[58] findings of a different promoter other than mechanical strain to trigger splicing of this gene. Nonetheless, the possibility of cellular swelling leading to mechanical transduction cannot be ruled out. It is also unknown whether there are individual variations in gene expression following damage and whether these variations in the concentrations seen in ovary correlate with a better or more deficient repair outcome.

Expression of both isoforms seen in the normal physiological response to ovulation and the pathological ovary showed a significant increase in the expression of both isoforms in the cancer samples (x100 fold increase). Microscopic evidence of this

difference of expression was easily appreciated by immune-analysis using polyclonal antibody for MGF. However, what are the consequences of this significant increase is unknown. Does MGF promote further differentiation of stem cells in tissues regardless of whether they are pathological (suffered a mutation) or normal? Does IGF-1Ea conduct its anabolic function on both normal and pathological tissue? These findings suggest that this autocrine repair mechanism is involved in tissue repair following physiological damage as it is the case of ovulation, as well as in the case of a pathological growth (ovarian cancer). Which agents may be responsible for activating the splicing of the IGF-1 gene in each case remains unknown. Whether there is a shift from primarily repair properties to a more proliferative pattern of this repair mechanism or the same repair mechanism is used indiscriminately in physiological or pathological conditions remains unknown. The increase in the expression of these isoforms in the pathological samples could reflect both a failure of this autocrine repair mechanism to restore damaged tissue or the reflection of an abnormal activation of these isoforms which contribute to the proliferation of abnormal cellular patterns present in the stem cells. Extensive literature supports the concept that the aethiology of ovarian cancer is multifactorial with genetic factors and protein

alterations of the tumour microenvironment leading to interference with apoptosis, and anti-growth signals allowing angiogenesis, invasion into stroma, and metastatic growth in distant organs. It remains a field for future research how the IGF-1 splice variants may interact within this microenvironment of matrix components, cell signalling pathways and inflammatory markers [137].

4.4.2. Expression of IGF-1 isoforms in the non gravid uterus

The uterus is the reproductive female organ that throughout premenopausal life is designed to bear the growing foetus. Multiple factors are involved in this growth and it is known that an increase in different female pregnancy hormones correlate with these growth changes. For this reason expression of IGF-1 splice variants in myometrium in the absence of injury to the muscle does not come as a complete surprise. During the gravid period, an organ measuring an average of 7 cm length will increase gradually to an average full term length of the body of the uterus of 40 cm. To achieve these changes it is easy to assume that the uterus will require of an autonomous mechanism which can provide new smooth muscle cells and promote anabolic growth. Further studies are needed to understand the implication of these splice variants

and their interaction with other agents to explain uterine growth during pregnancy.

In the present study, the expression of IGF-1 isoforms were seen not only in myometrium but also in uterine leiomyomas, one of the most common benign pathologies in gynaecological practice and the most common indication for hysterectomy (the three conditions most often associated with hysterectomy were uterine leiomyoma, endometriosis, and uterine prolapse) with around 600.000 hysterectomies were performed yearly in the USA between 2000 and 2004 [169]. However, the proportion of hysterectomies with an indication of uterine leiomyoma decreased significantly from 44.2% in 2000 to 38.7% in 2004.

Leiomyomas are the most common benign pelvic tumours in women and are present in 20 40% of women in their reproductive age [170]. They originate in the smooth muscular uterine tissue and vary in size from grain-sized ones to large uterine growths. They can be solitary and multiple and can be found in the different layers of the uterus. They are known to be oestrogen dependent tumours and their growth seems to correlate with the circulating exposure to estrogens. They decrease during menopause and other hypoestrogenic conditions, but tend to grow rapidly during

pregnancy. Clinical manifestations include menorrhagia (heavy blood loss with menses), dysmenorrhoea, pressure symptoms or sub-fertility. Disturbances in local growth factors and aberrant angiogenesis have also been involved in the aetiology of bleeding abnormalities [171].

In this study, samples were obtained from three patients suffering from leiomyomas who underwent a hysterectomy as treatment for their symptoms. The results show high expression of the two IGF-1 splice variants comparable to those seen on the LA muscle following delivery. As discussed previously, uterine leiomyomas are monoclonal tumours derived from a single myometrial cell mutation. The factors involved in their initiation and growth remain poorly understood but research to date suggests that the neoplastic transformation of myometrium to leiomyoma it is likely to involve somatic mutations of normal myometrium and the complex interactions of sex steroids with local growth factors. It is within this context that expression of MGF and IGF-1a may play a significant role. As seen in our previous work conducted in the ovary, MGF and IGF-1a appear to be directly involved in the proliferative growth of both pathological and non pathological tumours (cystoadenoma). Further studies are required with larger numbers where the interaction of these splice variants and the different agents involved

in leiomyoma growth can be studied. Despite the small numbers, it is not unreasonable to speculate that if the ultimate goal of this repair mechanism is to replace damaged tissues starting at stem cell level, detection of a damaged or abnormal myometrium cell could trigger this repair mechanism. What factors may promote the growth of abnormal proliferative tissue as seen in ovarian cancer shifting away from its primary repair function is still unknown. The difference of expression (highly intensified within the leiomyoma sample) of the targeted growth factors was also noticeable in the tissues undergoing immunostaining.

Interestingly, one of the most relevant findings of this group of patients were the samples obtained from a patient who following embolisation of the uterine artery underwent infarction of the fibroids and required an emergency hysterectomy to prevent further sepsis spreading from tissue necrosis. Uterine embolisation has its rational behind the uses of embolotherapy seen in other areas of medicine. Evidence shows that following embolisation leiomyomas experiment a decrease in size of the 50% within twelve months of the procedure. Complications such as the one described in this work are a rare occurrence and hence the relevance of the opportunistic sampling of these tissues. The samples obtained from the necrotized fibroid were unsuitable for analysis with RT-

PCR or immunostaining, However although the samples obtained from the leiomyomas embolised experienced necrosis, the myometrium usually preserves its blood supply partly through other pathways, keeping its viability. Comparison of MGF levels in the three myometrium specimens did not reveal any significant difference of expression. Contrary to this, IGF-1Ea appeared significantly reduced in the embolised sample. These findings suggest that whilst splicing of IGF-1 into MGF does take place at a local level (autocrine), the isoform IGF-1Ea is also present systemically and is available through this pathway. Restriction of blood supply via embolisation of the uterine artery will restrict access of IGF-1Ea to the leiomyoma bed, hence hampering the anabolic component of this repair mechanism. Although the fibroid growth is known to be hormone dependent, this is not the only mechanism involved as seen by the presence of other local growth factors in this process. Low levels of IGF-1Ea may play a determinant role in fibroid growth restriction following embolisation. Future research on the interaction between the different sex hormones and growth factors involved in fibroid growth and these IGF-1 splice variants may contribute to a better understanding of this condition and develop new therapies for the treatment of leiomyomas and their associated pathology.

4.5 Implications for future research

The present research has been able to demonstrate the presence of a muscle repair mechanism in the LA muscle which would be required under physiological conditions in order to restore function after injury. We have proven that during parturition, injury following vaginal delivery not only takes place at a macroscopic level as seen in several published imaging studies, but also takes place at a cellular level where this physiological mechanism is in place in order to repair the damaged tissues. How valuable and efficient this repair mechanism is in the presence of the physically torn muscle is still to be established. We also know that this physiological response may correlate in intensity depending on the different agents playing a role in vaginal delivery such as length of the second stage or type of delivery. Although more recent research, has established MGF s regeneration function in ex-vivo studies [172], it is still unknown whether MGF can be utilised as a marker of damage only, or as a marker of repair capability. We don t know yet, if this repair mechanism has variations in expression depending on ethnic group, mode of delivery or age at the time of delivery. It will also be of interest in the future to assess these repair markers comparing different obstetric scenarios such as

spontaneous vaginal delivery, instrumental delivery, or emergency caesarean section between them and with the ideal control being elective caesarean section. In the long term, it would be of interest to determine the anatomical configuration and pelvic floor function after delivery in relation with the expression of these repair factors. Longitudinal studies looking at the correlation between LA muscle injury as seen using transperineal ultrasound, pelvic floor pathology evaluated using validated questionnaires is an area of research for the future. The role played by these repair factors in combination with the two premises above would give a more in depth understanding of the complexities of pelvic floor injury following vaginal delivery.

We have also demonstrated that this repair mechanism, initially thought to be present only in skeletal muscle, it may also be taking place in other organs of human body, and in particular in the female reproductive system. Further research is needed to establish what agents may trigger the splicing of the IGF-1 gene. It has been assumed in previous experiments that mostly straining or chemical injury were the main external agents leading to the splicing of the IGF-1 gene, however our findings showing expression of the two splice variants in ovary and myometrium suggest other mediators as triggers of this splicing. This is not entirely unexpected as the

literature had already documented the expression of the IGF-1 gene before in both ovary and leiomyomas. What is innovative about our findings is that it could explain the mechanism by which the ovary repairs its epithelium following ovulation, and in the presence of neoplastic cells, how it fails to do so. Of interest, our findings regarding the expression of MGF on cancerous tissues have recently been supported by the findings of Armakolas et al in cancerous tissues of the human prostate [173].

It is also of interest the direct involvement of the studied splice variants in the development of benign tumours as seen in the non gravid uterus and it remains an area for research how these peptides may interact with other known growth factors involved in fibroid growth. More recently expression of MGF has already been documented in eutopic and ectopic endometrium. The role played by MGF in the process of endometrium regeneration and associated pathology remains an area for research in the future [174]. Finally, it may be also a subject of interest to study the involvement of this repair mechanism in the gravid uterus and how it may contribute to generate new smooth muscle cells from progenitor cells and promote further hyperplasia.

But more importantly, this has been the first time these splice variants have been shown to play a role in smooth muscle

regeneration and it will be an interesting next step to understand how this repair mechanism may contribute to tissue regeneration in organs with predominantly smooth muscle submitted also to distension forces such as blood vessels, bowel and bladder.

Since the completion of this thesis the author has conducted several experiments in the human bladder, demonstrating for the first time the expression of these repair factors both in detrusor muscle and urothelium. The implication of these findings to understand the pathophysiology of the bladder and how therapies may work, is a line for future research [175-6].

APPENDIX 1

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Dr Cortes
Department of Obstetrics and Gynaecology

Dear Dr Euward Cortes,

Re: MRI changes of levator ANI during pregnancy

Project ID: 5705

I have received another favourable scientific peer review for the above project. The reviewer raised a few issues concerning the project and for your information I have attached an extract of their comments.

Yours sincerely,

[redacted]
Zoë Spyvee

R&D Officer

APPENDIX 2

Study group biopsy

Picture showing a patient following first spontaneous vaginal delivery. The vaginal tear seen extends vertically 3-4 cm on the right postero- lateral aspect of the vaginal wall, showing direct access to the pubo-rectalis component of the LA muscle .

APPENDIX 3

Cutting needle biopsy (Monoject, ABCâ)

Control group biopsy

Insertion of the cutting needle biopsy in a patient having gynaecological surgery not related to pelvic floor disorders.
The cutting needle biopsy is inserted 2 to 3 cm in the right postero-lateral aspect of the vagina, following digital guidance, into the right aspect of the pubo-rectalis component of the LA muscle.

Appendix 4

Search History

1. MEDLINE; "levator ani muscle".ti,ab; 493 results.
11. MEDLINE; "Mechano growth factor".ti,ab; 95 results.
12. MEDLINE; 10 OR 11; 627 results.
13. MEDLINE; 9 OR 12; 47198 results.
14. MEDLINE; 1 AND 13; 8 results.
15. MEDLINE; "insulin-like growth factor*".ti,ab; 32808 results.
10. MEDLINE; "MGF".ti,ab; 611 results.
17. MEDLINE; 12 OR 16; 47700 results.
18. MEDLINE; 1 AND 17; 8 results.
19. EMBASE; "levator ani muscle".ti,ab; 582 results.
20. EMBASE; "IGF".ti,ab; 38903 results.
21. EMBASE; IGF-1Ec.ti,ab; 17 results.
8. MEDLINE; "Insulin-like growth factor".ti,ab; 31034 results.
2. MEDLINE; "IGF".ti,ab; 33988 results.
4. MEDLINE; IGF*.ti,ab; 39448 results.
9. MEDLINE; 2 OR 7 OR 8; 46685 results.
16. MEDLINE; 9 OR 15; 47187 results.
22. EMBASE; IGF*.ti,ab; 45928 results.
29. EMBASE; "Mechano growth factor".ti,ab; 98 results.
28. EMBASE; "MGF".ti,ab; 530 results.
27. EMBASE; 20 OR 25 OR 26; 58431 results.
23. EMBASE; IGF-1Ea.ti,ab; 26 results.
33. EMBASE; "insulin-like growth factor*".ti,ab; 36279 results.
34. EMBASE; 27 OR 33; 59046 results.
35. EMBASE; 30 OR 34; 59464 results.
36. EMBASE; 19 AND 35; 8 results.
25. EMBASE; INSULIN-LIKE GROWTH FACTOR I/; 36545 results.
38. EMBASE; 19 [Limit to: Publication Year 2007-2011]; 211 results.
32. EMBASE; 19 AND 31; 8 results.
26. EMBASE; "Insulin-like growth factor".ti,ab; 34230 results.
31. EMBASE; 27 OR 30; 58849 results.
37. MEDLINE; 1 [Limit to: Publication Year 2007-2011]; 134 results.
24. EMBASE; IGF1Ea.ti,ab; 5 results.
6. MEDLINE; IGF1Ea.ti,ab; 3 results.
30. EMBASE; 28 OR 29; 546 results.
7. MEDLINE; INSULIN-LIKE GROWTH FACTOR I/; 27283 results.
3. MEDLINE; IGF-1Ec.ti,ab; 14 results.
5. MEDLINE; IGF-1Ea.ti,ab; 22 results.
39. MEDLINE; "levator ani muscle".ti,ab; 493 results.
46. MEDLINE; "Insulin-like growth factor".ti,ab; 31034 results.
40. MEDLINE; "IGF".ti,ab; 33988 results.
45. MEDLINE; INSULIN-LIKE GROWTH FACTOR I/; 27283 results.
41. MEDLINE; IGF-1Ec.ti,ab; 14 results.
50. MEDLINE; 48 OR 49; 627 results.
54. MEDLINE; 47 OR 53; 47187 results.
51. MEDLINE; 47 OR 50; 47198 results.

43. MEDLINE; IGF-1Ea.ti,ab; 22 results.
57. EMBASE; "levator ani muscle".ti,ab; 582 results.
58. EMBASE; "IGF".ti,ab; 38903 results.
59. EMBASE; IGF-1Ec.ti,ab; 17 results.
60. EMBASE; IGF*.ti,ab; 45928 results.
61. EMBASE; IGF-1Ea.ti,ab; 26 results.
62. EMBASE; IGF1Ea.ti,ab; 5 results.
63. EMBASE; INSULIN-LIKE GROWTH FACTOR I/; 36545 results.
64. EMBASE; "Insulin-like growth factor".ti,ab; 34230 results.
65. EMBASE; 58 OR 63 OR 64; 58431 results.
55. MEDLINE; 50 OR 54; 47700 results.
48. MEDLINE; "MGF".ti,ab; 611 results.
52. MEDLINE; 39 AND 51; 8 results.
69. EMBASE; 65 OR 68; 58849 results.
67. EMBASE; "Mechano growth factor".ti,ab; 98 results.
53. MEDLINE; "insulin-like growth factor*".ti,ab; 32808 results.
72. EMBASE; 65 OR 71; 59046 results.
56. MEDLINE; 39 AND 55; 8 results.
66. EMBASE; "MGF".ti,ab; 530 results.
75. MEDLINE; 39 [Limit to: Publication Year 2007-2011]; 134 results.
76. EMBASE; 57 [Limit to: Publication Year 2007-2011]; 211 results.
77. MEDLINE; "levator ani muscle".ti,ab; 493 results.
78. MEDLINE; "IGF".ti,ab; 33988 results.
79. MEDLINE; IGF-1Ec.ti,ab; 14 results.
49. MEDLINE; "Mechano growth factor".ti,ab; 95 results.
81. MEDLINE; IGF-1Ea.ti,ab; 22 results.
68. EMBASE; 66 OR 67; 546 results.
83. MEDLINE; INSULIN-LIKE GROWTH FACTOR I/; 27283 results.
84. MEDLINE; "Insulin-like growth factor".ti,ab; 31034 results.
73. EMBASE; 68 OR 72; 59464 results.
86. MEDLINE; "MGF".ti,ab; 611 results.
87. MEDLINE; "Mechano growth factor".ti,ab; 95 results.
42. MEDLINE; IGF*.ti,ab; 39448 results.
89. MEDLINE; 85 OR 88; 47198 results.
90. MEDLINE; 77 AND 89; 8 results.
91. MEDLINE; "insulin-like growth factor*".ti,ab; 32808 results.
74. EMBASE; 57 AND 73; 8 results.
44. MEDLINE; IGF1Ea.ti,ab; 3 results.
85. MEDLINE; 78 OR 83 OR 84; 46685 results.
70. EMBASE; 57 AND 69; 8 results.
96. EMBASE; "IGF".ti,ab; 38903 results.
97. EMBASE; IGF-1Ec.ti,ab; 17 results.
95. EMBASE; "levator ani muscle".ti,ab; 582 results.
99. EMBASE; IGF-1Ea.ti,ab; 26 results.
100. EMBASE; IGF1Ea.ti,ab; 5 results.
82. MEDLINE; IGF1Ea.ti,ab; 3 results.
102. EMBASE; "Insulin-like growth factor".ti,ab; 34230 results.
101. EMBASE; INSULIN-LIKE GROWTH FACTOR I/; 36545 results.
104. EMBASE; "MGF".ti,ab; 530 results.
105. EMBASE; "Mechano growth factor".ti,ab; 98 results.
103. EMBASE; 96 OR 101 OR 102; 58431 results.

107. EMBASE; 103 OR 106; 58849 results.
108. EMBASE; 95 AND 107; 8 results.
92. MEDLINE; 85 OR 91; 47187 results.
94. MEDLINE; 77 AND 93; 8 results.
111. EMBASE; 106 OR 110; 59464 results.
112. EMBASE; 95 AND 111; 8 results.
113. MEDLINE; 77 [Limit to: Publication Year 2007-2011]; 134 results.
114. EMBASE; 95 [Limit to: Publication Year 2007-2011]; 211 results.
93. MEDLINE; 88 OR 92; 47700 results.
116. MEDLINE; 39 AND 115; 16 results.
117. MEDLINE; (levator AND ani AND injury).ti,ab; 62 results.
118. MEDLINE; 115 AND 117; 30 results.
119. MEDLINE; 87 AND 117; 0 results.
110. EMBASE; 103 OR 109; 59046 results.
109. EMBASE; "insulin-like growth factor*".ti,ab; 36279 results.
106. EMBASE; 104 OR 105; 546 results.
47. MEDLINE; 40 OR 45 OR 46; 46685 results.
120. MEDLINE; 48 AND 117; 0 results.
121. MEDLINE; (IGF AND splice AND variants).ti,ab; 49 results.
98. EMBASE; IGF*.ti,ab; 45928 results.
80. MEDLINE; IGF*.ti,ab; 39448 results.
122. MEDLINE; 117 AND 121; 0 results.
71. EMBASE; "insulin-like growth factor*".ti,ab; 36279 results.
88. MEDLINE; 86 OR 87; 627 results.
115. MEDLINE; (pelvic AND floor AND injury).ti,ab; 267 results.
124. MEDLINE; "levator ani muscle".ti,ab; 493 results.
125. MEDLINE; "IGF".ti,ab; 33988 results.
135. MEDLINE; 132 OR 133; 627 results.
127. MEDLINE; IGF*.ti,ab; 39448 results.
129. MEDLINE; IGF1Ea.ti,ab; 3 results.
137. MEDLINE; 123 AND 135; 8 results.
130. MEDLINE; INSULIN-LIKE GROWTH FACTOR I/; 27283 results.
138. MEDLINE; "insulin-like growth factor*".ti,ab; 32808 results.
141. MEDLINE; 123 AND 139; 8 results.
131. MEDLINE; "Insulin-like growth factor".ti,ab; 31034 results.
143. EMBASE; "IGF".ti,ab; 38903 results.
144. EMBASE; IGF-1Ec.ti,ab; 17 results.
145. EMBASE; IGF*.ti,ab; 45928 results.
146. EMBASE; IGF-1Ea.ti,ab; 26 results.
134. MEDLINE; "Mechano growth factor".ti,ab; 95 results.
136. MEDLINE; 131 OR 134; 47198 results.
149. EMBASE; "Insulin-like growth factor".ti,ab; 34230 results.
150. EMBASE; 142 OR 147 OR 148; 58431 results.
140. MEDLINE; 134 OR 138; 47700 results.
126. MEDLINE; IGF-1Ec.ti,ab; 14 results.
128. MEDLINE; IGF-1Ea.ti,ab; 22 results.
154. EMBASE; 149 OR 152; 58849 results.
132. MEDLINE; 124 OR 129 OR 130; 46685 results.
148. EMBASE; INSULIN-LIKE GROWTH FACTOR I/; 36545 results.
152. EMBASE; "Mechano growth factor".ti,ab; 98 results.
147. EMBASE; IGF1Ea.ti,ab; 5 results.

159. EMBASE; 141 AND 157; 8 results.
160. MEDLINE; 123 [Limit to: Publication Year 2007-2011]; 134 results.
151. EMBASE; "MGF".ti,ab; 530 results.
153. EMBASE; 150 OR 151; 546 results.
133. MEDLINE; "MGF".ti,ab; 611 results.
156. EMBASE; "insulin-like growth factor*".ti,ab; 36279 results.
158. EMBASE; 152 OR 156; 59464 results.
155. EMBASE; 141 AND 153; 8 results.
166. EMBASE; 143 AND 150 AND 151 AND 162; 0 results.
168. MEDLINE; 150 OR 151 OR 162; 689 results.
169. EMBASE; (pelvic AND floor AND injury).ti,ab; 418 results.
167. EMBASE; 150 OR 151 OR 162; 632 results.
161. EMBASE; 141 [Limit to: Publication Year 2007-2011]; 211 results.
157. EMBASE; 149 OR 155; 59046 results.
173. MEDLINE; 123 AND 130 AND 133 AND 137 AND 164 AND 169; 0 results.
174. MEDLINE; 123 AND 129 AND 130 AND 164; 0 results.
175. MEDLINE; 123 AND 164; 0 results.
176. MEDLINE; 123 AND 169; 16 results.
177. MEDLINE; 123 AND 169; 16 results.
178. MEDLINE; 123 AND 129 AND 130; 1 results.
164. EMBASE; (IGF-1 AND splice AND variants).ti,ab; 16 results.
171. EMBASE; 141 AND 143 AND 151 AND 155 AND 162 AND 163 AND 168; 0 results.
172. MEDLINE; 125 AND 133 AND 137 AND 161 AND 164 AND 169; 0 results.
170. MEDLINE; (pelvic AND floor AND injury).ti,ab; 267 results.
139. MEDLINE; 131 OR 137; 47187 results.
142. EMBASE; "levator ani muscle".ti,ab; 582 results.
162. MEDLINE; (levator AND ani AND injury).ti,ab; 62 results.
163. EMBASE; (levator AND ani AND injury).ti,ab; 86 results.
165. MEDLINE; (IGF-1 AND splice AND variants).ti,ab; 13 results.
179. MEDLINE; (obstetric AND injury).ti,ab; 798 results.
180. MEDLINE; 178 AND 179; 0 results.
181. MEDLINE; (vaginal AND delivery).ti,ab; 11512 results.
182. MEDLINE; 178 AND 181; 1 results.
183. MEDLINE; (spontaneous AND vaginal AND delivery).ti,ab; 1766 results.
184. MEDLINE; 178 AND 183; 0 results.
185. MEDLINE; (second AND stage AND of AND labour).ti,ab; 702 results.
186. MEDLINE; 178 AND 185; 0 results.

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Publications and presentations related to this research

1. **Correlation of clinical examination and MR imaging in vault prolapse**.
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2. **Clinical Examination and Dynamic Magnetic Resonance Imaging in Vaginal Vault Prolapse.**
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3. **Insulin-like growth factor - 1 (IGF-1) gene splice variants as markers of muscle damage in levator ani (LA) muscle following first vaginal delivery.**

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4. **Expression of IGF-1 splice variants in the Levator ani muscle in young and elderly women. Why pelvic floor exercises fail in postmenopausal women?**
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6. **Expression of Insulin Growth Factor (IGF) splice variants (IGF-1a and MGF) in myometrium and leiomyomas. Does fibroid growth follow muscle damage?**

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