An Ultrastructural and Immunocytochemical Study of Myometrium and its Leiomyomata

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Thesis presented for the degree of

Doctor of Philosophy

in the Department of Anatomical Pathology

University of Cape Town

August 1995

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Peter

For your endurance, encouragement and sagacity.
My thanks and love.

ACKNOWLEDGMENTS

I wish to acknowledge my indebtedness to the many people who have helped accomplish a task that I could not have completed alone. In particular I should like to thank:

Professor Andrew J. Tiltman, Associate Professor of the Department of Anatomical Pathology, University of Cape Town, under whose supervision this work was conducted. His encouragement, insight and enthusiasm made completing this thesis a pleasure.

For their assistance and excellent instruction in all matters technical, in alphabetical order:

Mrs. S. Cornelius, Mr M. Hendricks, Mrs. H. Ilsley, Ms H. Rees, Mr. P. Richards, Ms M. Schinkel, Mrs. L. Scott, Ms B. Seymour, Mr. W. Williams.

Special thanks to Mrs. E. Fuller, Department of Anatomy and Cell Biology, University of Cape Town for the superb medical illustrations to be found in this thesis. Finally, to my husband Peter for the endless hours of debate, proofreading and tea making.

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ABSTRACT

Leiomyomata are benign tumours that occur in the female genital tract with great frequency. These tumours are by no means innocuous and complications related to their presence form a major percentage of gynaecological morbidity with substantial financial implications for both health services and patients.

Even though leiomyomata have received a great deal of scientific attention, their aetiology remains unknown. To this end, this thesis was aimed at gaining a better understanding of the fundamental character of the tumours and the myometrium in which they occur. Three basic questions were asked. Firstly, whether or not the non-neoplastic myometrium of leiomyomatous uteri is normal. Secondly, what structural and oestrogen receptor differences exist between normal myometrium and leiomyomata and thirdly, whether the tumours and normal myometrium respond in a like manner to changes in age, parity and endogenous hormone.

Samples of myometrial tissue and when present, leiomyomata, were taken from a total of 191 surgically resected uteri. The tissue was processed for electron microscopy, radioimmunoassay and immunocytochemistry using standard methodologies. Blood collected from the patients at the time of surgery was used for the measurement of serum estradiol, progesterone and sex hormone binding globulin.

Leiomyomatous myometria cannot be considered normal as firstly, they show a possible ultrastructural increase in plasmalemmal densities with a concomitant decrease in vesicles. Secondly, leiomyomatous myometria differ significantly from normal myometria with respect to their oestrogen receptor content. They demonstrate higher levels of the receptor with flattening out of the receptor distribution curve.

Leiomyomata are distinct entities with oestrogen receptor contents far higher than that of the mean for normal myometrium but similar to those of their host myometrium. Ultrastructurally the tumours are very similar to the myometrium in which they occur. The presence of a population of myofibrocytes within the tumours lends credence to theories of unicellular origin and cellular pleuripotentiality.

Both normal myometrium and leiomyomata respond in a similar fashion to changes in endogenous oestrogen in that neither undergoes ultrastructural or receptor content alteration. Leiomyomata cannot be dated in the same terms as normal myometrium. The aged myometrium shows an increase in the amount of lipofuscin and a decrease in lipid-rich residual bodies. Larger leiomyomata are probably 'older' than the smaller ones and usually appear more fibrous.

That leiomyomatous myometrium is not normal has major implications with regard to the manner in which research into leiomyomata is conducted. The finding also suggests that leiomyomata may well arise due to a primary subcellular abnormality of the myometrium in which they occur.

PREFACE

How strange it is that some of the most common place events are those which defy explanation. This is often the case within medicine and its associated sciences, for despite major advances in technology and an increasing body of knowledge, definitive answers to the prevention and cure for common place events such as the annual 'cold' continue to elude us.

Within the speciality of gynaecological pathology, the issue of leiomyomata, or fibroids, provides an analogous situation. This benign tumour is known to occur within the female genital tract with great frequency. Despite their commonality, the etiology and definitive medical and surgical management of leiomyomata remains the subject of active investigation and debate.

The purpose of this thesis is to address leiomyomata on two fronts. Firstly, an ultrastructural study of both normal and leiomyomatous uteri, in the hope of establishing base lines for structural differences between normal and abnormal tissue, as well as examining such tissues for their structural response to differences in endogenous oestrogen, parity and age.

Secondly, as structure and function are intimate reflections of one another, a basic functional analysis of myometrium and its leiomyomata is undertaken. The oestrogen receptor status of normal, leiomyomatous myometrium and leiomyomata is examined, as well as any changes in the receptor status in response to variations in endogenous oestrogen.

Thus, in summary, the questions this thesis aims to answer are:

1) Is the non-neoplastic myometrium of leiomyomatous uteri normal or abnormal?

2) What differences in structure and oestrogen receptor content are there between normal myometrium and leiomyomata?

3) Do leiomyomata and myometrium respond in a like manner to changes in age, parity and endogenous oestrogen?

CHAPTER ONE

THE NORMAL UTERUS

1) UTERINE GROSS ANATOMY

1.1) *Peritoneal Reflections and General Relations*

The uterus (Figures 1 and 2) is normally situated in the lower pelvis where it lies anterior to the rectum and posterior to the urinary bladder. It is covered both anteriorly and posteriorly by reflections of the pelvic peritoneum. Anteriorly, at the level of the internal cervical os, the peritoneum is reflected onto the bladder as the uterovesical fold thus forming the vesicouterine pouch. The peritoneum covering the posterior surface of the uterus extends down to the cervix and upper vagina, prior to reflecting onto the rectum to form the rectouterine pouch (Anthony & Thibodeau 1983).

The anterior and posterior peritoneal reflections continue laterally forming the anterior and posterior leaves of the uterine broad ligaments. These tent-like broad ligaments house the major uterine vessels as well as the efferent lymphatic trunks. The fallopian tubes are located in the free edge of each broad ligament. Situated postero-lateral and inferior to the utero-tubal junctions are the utero-ovarian ligaments which attach the ovaries to the ipsilateral uterine cornua. The round ligaments arise anteroinferiorly to the attachment of the fallopian tubes to the fundus and run between the layers of the broad ligament across the wall to the deep inguinal ring. They traverse the inguinal canal and merge with the subcutaneous tissues of the labium majorum (Williams & Warwick 1984).

The body of the uterus, enclosed within the layers of the broad ligament, is freely movable while the cervix has limited mobility as it is anchored to its surroundings by a number of connective tissue bands, including the transverse cervical and uterosacral ligaments. The pelvic floor and pelvic viscera form the principle supports of the uterus (Williams & Warwick 1984).

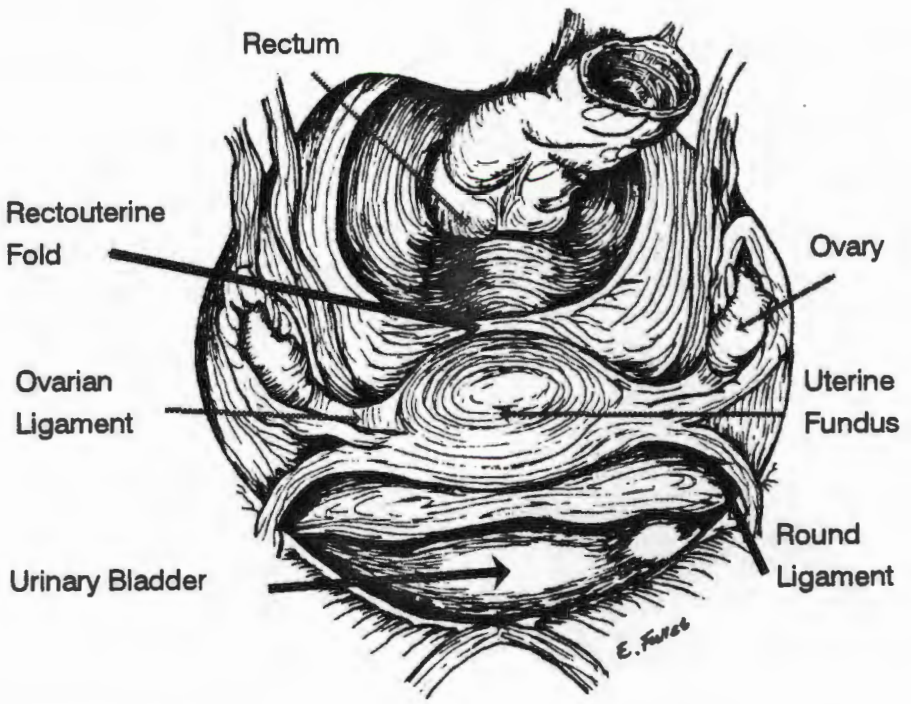

Figure 1: Schematic representation of the female pelvic contents as seen from above.

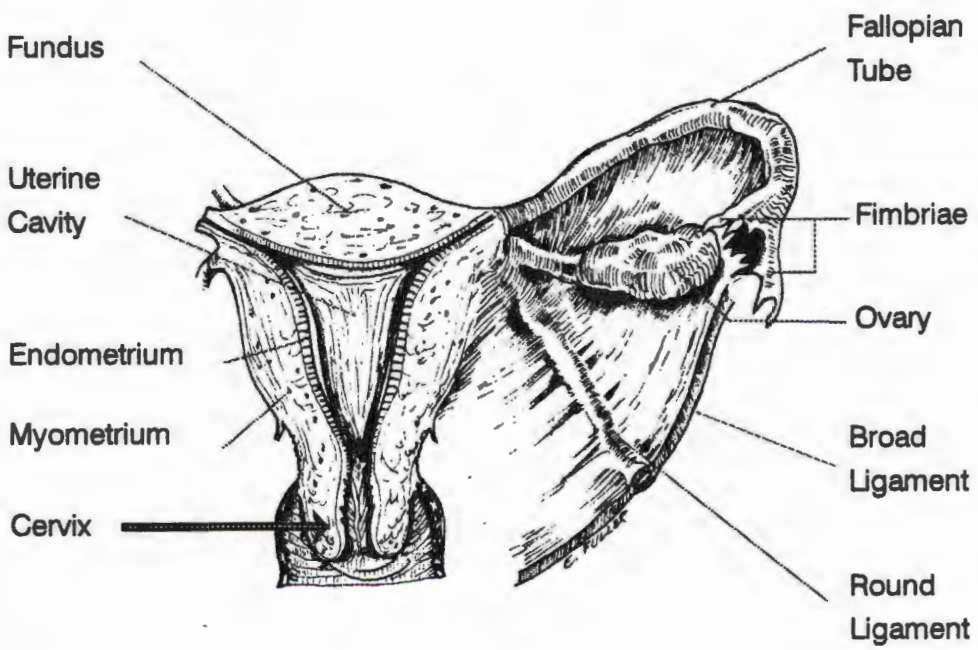

Figure 2: Schematic representation of the female genital organs, posterior view.

1.2) *Gross Anatomical Features*

The adult nulliparous uterus is described as a pear-shaped, thick walled, hollow organ with an average weight of 40 to 80 grams. It usually measures 7 to 8 cm along its long axis, 5cm from cornu to cornu, and approximately 2,5cm in the antero-posterior plane (Williams & Warwick 1984). The uterus is divisible into two major portions, the expanded superior two thirds termed the corpus or body and the smaller cylindrical inferior third, the cervix.

The fundus of the corpus is that portion located cephalic to an imaginary line joining the points of entrance of the two fallopian tubes. The cornua or horns are the two lateral regions of the fundus that are associated with the intramural portions of the fallopian tubes. The corpus tapers from the fundus to become the isthmus or lower uterine segment. This region forms a narrow transition zone between the uterine body and the cervix (Hendrickson & Kempson 1992).

The uterine cavity is of the approximate configuration of the uterus but due to the substantial thickness of the uterine wall it is of much smaller dimensions. The apices of this triangular potential space are continuous with the lumina of the fallopian tubes as well as with the endocervical canal at the level of the internal os.

Inferior to the corpus lies the cervix, a roughly cylindrical structure measured sonographically to be about 3 to 4cm in length (Zemlyn 1981). The cervix is pierced through its centre by the endocervical canal. This canal has an external os, opening onto the exocervix and an internal os which communicates with the main uterine cavity.

1.3) *The Uterine wall*

The uterine wall is divided into three morphologically distinct layers:

* The external serosa (perimetrium)
* The muscular layer (myometrium)
* The mucosa (endometrium)

The external serosa forms an outer serous coat and consists of peritoneum supported by a thin layer of connective tissue.

The myometrium forms most of the uterine wall, measuring about 1,25cm in thickness at the fundus and mid-level but tapering at the tubal orifices. It consists of bundles of smooth muscle cells in an admix of loose connective tissue, blood vessels, lymph vessels and nerves.

Although the smooth muscle cells of the myometrium interface in all directions, they form three layers of variable distinction, the internal, middle and external layers. The muscle cells of the internal submucosal layer are arranged in both longitudinal and circular patterns and are related to the deep parts of the endometrial glands. The thick intermediate or middle layer contains randomly interdigitating fibres running in all planes and is richly populated with larger blood vessels. The fibres of the external layer tend to be predominantly longitudinal, with the fibres passing over the fundus, converging at the lateral angles and continuing into the uterine tubes.

Two lateral, subserosally situated, longitudinally arranged, bands of muscle fibres, the fasciculi cervicoangulares, have been described (Toth & Toth 1974). These bundles bridge the cervix and corpus uteri, having well defined boundaries and a clear longitudinal course. The identification of epithelial remnants, resembling Wolffian duct structures, within these bundles is suggestive of their having arisen from the longitudinal muscle coat of the mesonephric ducts (Toth 1977). As the fasciculi cervicoangulares are the only longitudinal muscle bundles that directly connect the cervix and fundus it has been postulated that they may serve as a conduction or co-ordinating system.

The endometrium, a mucosal layer, is continuous via the uterine tubes with the peritoneal cavity and via the external cervical os with the vaginal mucosa. The endometrium is subject to cyclical changes under the influence of the reproductive steroid hormones.

Magnetic resonance imaging of the normal myometrium and endometrium demonstrates that minor changes in the total uterine volume occur during the menstrual cycle, with the greatest volume been attained during the secretory phase (Hricak 1986).

1.4) *Uterine Vasculature*

The arterial supply of the myometrium is derived from the left and right uterine arteries which arise as branches of the internal iliac arteries. At the level of the uterine isthmus, the uterine arteries divide into ascending and descending branches. The ascending uterine artery anastomoses with the ovarian arterial supply while the descending artery anastomoses with the vaginal arterial supply. Both the descending and ascending arteries give rise to a network of circumferentially arranged vessels, the arcuate arteries. The arcuate arteries supply multiple tortuous radial branches which penetrate and supply the myometrium in the form of dense capillary networks (Farrer-Brown *et al* 1970a). In the inner third of the myometrium the radial arteries branch to form spiral arteries which in turn supply the endometrium. The venous drainage parallels the supply, although the vessels are volumetrically greater. As is to be expected all the vessels within the uterus are markedly tortuous in order to accommodate vast changes in uterine size and shape during the reproductive years.

Multiple lymphatic vessels are present in the corpus and cervix. In the myometrium they form a complex labyrinth which courses to a submucosal plexus. The channels formed from these plexuses ramify over the entire surface of the uterus and converge to form the major lymphatic trunks of the uterus. The cervix is subsequently drained via the external and internal iliac nodes as well as via the rectal and sacral nodes. The lower part of the uterine body is drained via the external iliac nodes while the lymphatic vessels from the upper part of the uterus pass to the lateral aortic and pre-aortic nodes.

1.5) *Innervation*

The uterus is innervated by both autonomic and sensory fibres via the uterovaginal plexi. These nerves course through the uterus with the uterine arteries. The uterus is supposedly insensitive to most pain stimuli however the cervix is often sensitive when grasped with forceps or dilated. Dysmenorrhea may be attributed to nerve fibres that ascend to enter the spinal cord via the splanchnic nerves.

2) MYOMETRIAL HISTOLOGY

The smooth muscle of the myometrium consists of typical blunt-ended, spindle-shaped cells with central fusiform nuclei. These muscle cells tend to range in length from 20μm during the non-pregnant state to upwards of 600μm in the term uterus (Schoenberg 1977). Cytoplasmic volume also varies according to whether or not the uterus is gravid. Between the interlacing bundles of muscle cells are varying amounts of collagenous fibres, fibroblasts, macrophages and mast cells (Figures 3 and 4). Within the peripheral layers of the uterine wall are numerous elastic fibre networks. These extend centrally between the muscle bundles but do not appear to breach the innermost layers of the myometrium.

The corpus of the uterus has a greater concentration of smooth muscle relative to the collagen and elastin when compared to that of the lower uterine segment and cervix. This distribution is consistent with the passive role of the cervix during parturition, during which the uterine contents are expelled by strong fundal contractions through a passively dilated cervix which is softened by the action of collagenase (Hendrickson & Kempson 1992).

3) MYOMETRIAL ULTRASTRUCTURE

3.1) *General*

The myocytes of the myometrium are similar in structure to most other smooth muscle cells, but differ functionally in that they are capable of responding to prostaglandin hormonal stimuli. Ultrastructurally (Figure 5) the cellular features reflect the dual function of these cells, i.e. :

* The contraction of the uterus
* The synthesis of collagen and elastin

3.2) *The Nucleus*

The nucleus usually presents as a centrally placed structure of irregular outline. It is bounded by a double nuclear membrane measured by Mark (1956) to be approximately 20nm thick. While the external margin of this membrane is distinct, the internal margin is usually obscured by a layer of heterochromatin. The nuclear matrix is composed of predominantly dense heterochromatin, most of which

is peripherally situated. The remainder of the nuclear matrix is amorphous in character (Ferenczy *et al* 1971). One or two nucleoli are commonly seen and tend to lie in the middle third of the nucleus (Mark 1956).

3.3) *Myofilaments*

Most of the cytoplasm of the myometrial myocytes is occupied by myofilaments. These filaments are arranged parallel to the long axis of the myocyte, maintaining their orientation through to the tapered ends of the cell. As each individual myofilament rapidly leaves the plane of section, they can rarely be traced for any great distance and accurate measurement of their length is thus difficult. The fact that the filaments maintain their orientation until they terminate obliquely on approaching the cell border, is suggestive that the central filaments may well be longer than those of the periphery (Mark 1956). In Mark's (1956) electron microscopic study of uterine smooth muscle he confirmed the work of earlier histologists who had noted that the myocytes of smooth muscle differed from those of striated muscle in having no constant interfilamentary distance and therefore no cross-striations.

Actin constitutes the majority of the myofilaments with an average individual diameter of 5 to 6nm. When viewed, in cross-section, at high magnification the filaments are packed in hexagonal or square arrays. Up to 100 filaments have been recorded within such an array (Lowy & Small 1970). Lowy *et al* (1970) used X-Ray diffraction studies to demonstrate the presence of filamentous smooth muscle myosin. These myosin filaments appear as long ribbon-like structures of 10 to 20nm in width and are orientated parallel to the adjacent actin filaments (Lowy & Small 1970).

The ratio of actin to myosin has been noted to be greater than that of striated muscle, with up to 15 actin filaments to every myosin filament (Schoenberg 1977). The presence of this dual filament system and its spatial orientation is suggestive of a sliding mechanism of contraction, not unlike that of skeletal muscle (Panner & Honig 1967, Rice *et al* 1970) and allows for the forces of contraction to be evenly spread through the cell rather than concentrated at its ends (Mark 1956).

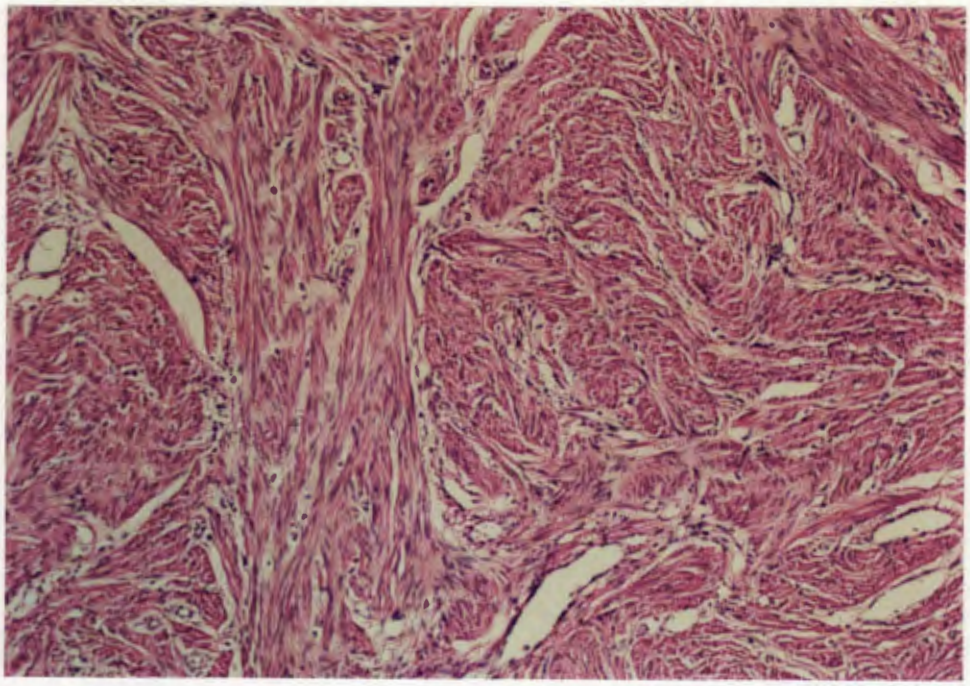

Figure 3: Light micrograph of a H&E stained section of normal myometrium from the fundal region. Note the longitudinal and transverse sectioning of the myocytes. Print magnification x110.

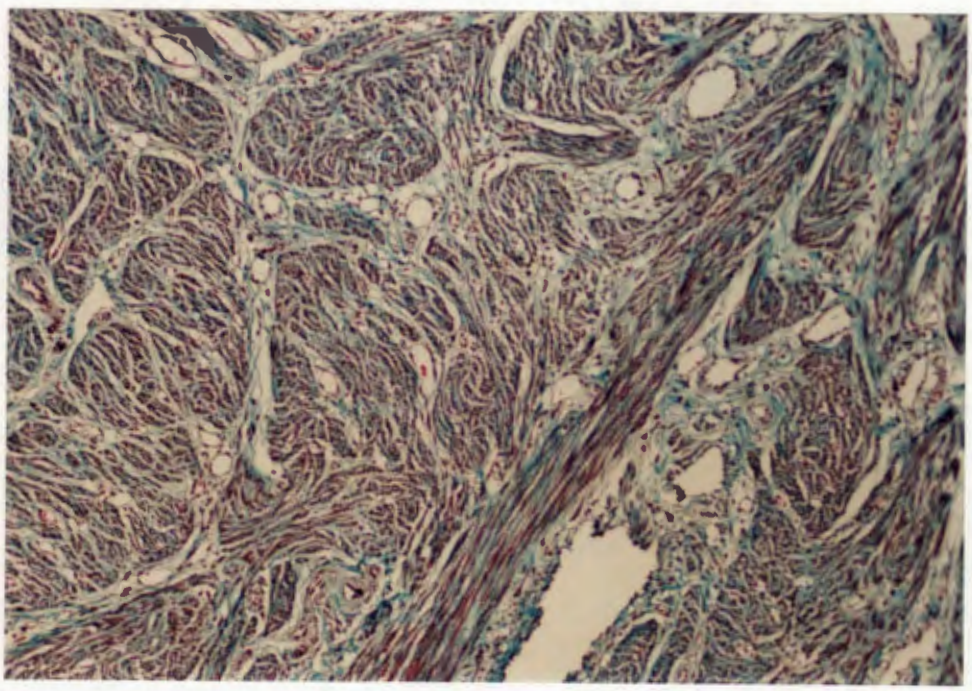

Figure 4: Light micrograph of a Masson's trichrome stained section of normal myometrium from the fundal region. Note the distribution of collagen (green) to muscle (red). Print magnification x110.

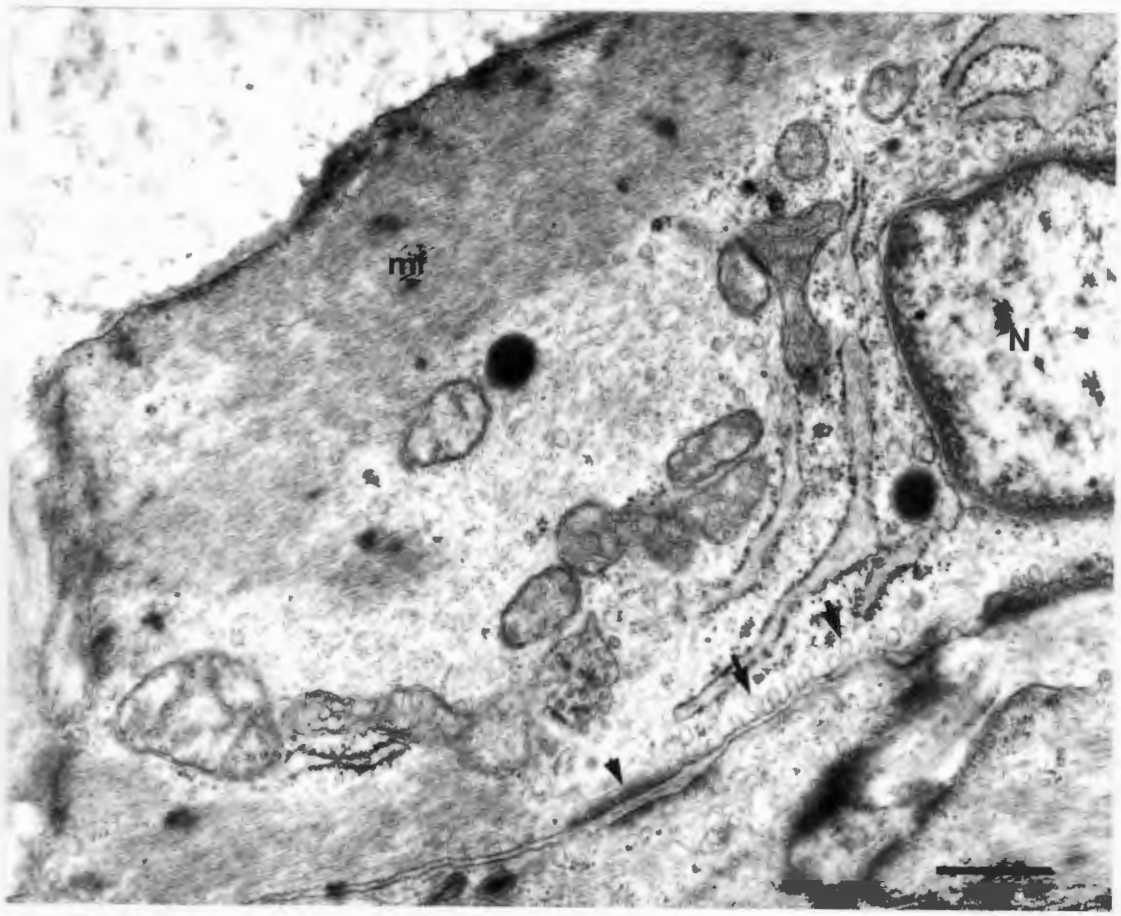

Figure 5: Electron micrograph of a longtitudinal section through a normal myocyte. The nucleus (N) is seen on the right hand side of the micrograph with prominant perinuclear organelles. Myofilaments (mf) are peripherally situated in the myocyte. Numerous small vesicles (arrows) can be seen on the plasmalemma along with distinct plasmalemmal dense bodies (arrow head). Scale bar = 0,5μm.

Along the trajectory of the filaments are numerous small electron dense bodies with their long axes parallel to the long axis of the cell. They are about 1μm long and 0,2 to 0,5μm in diameter, with no specific order or spacing. Numbers of similar dense plaques are located along the inner aspect of the plasma membrane. It was originally suggested that these plaques may be small contracted portions of bundles of the filaments or alternatively chance crossings of a number of filaments. These dense bodies were, at one stage, considered to be artifactual (Mark 1956). Panner and Honig (1967) note that the focal densities as well as the plasmalemmal densities are composed of 2nm thick actin filaments with collateral thick filament attachments. A few myosin filaments have been identified within these densities. It has been suggested that as the densities provide a form of attachment for the myofilaments, that they may be considered to be analogous to the z-lines of the striated muscle cell (Ferenczy 1979).

3.4) *Organelles and Inclusions*

Myocytes contain the same set of organelles and inclusions as those found in other forms of smooth muscle. These include smooth and rough sarcoplasmic reticulum, surface vesicles, golgi apparatus and numerous mitochondria. Most of the organelles, which tend to be relatively scant, are located at the nuclear poles.

The sarcoplasmic reticulum of the myocyte is not usually as well developed as that of skeletal muscle cells and the amount of reticulum varies according to whether the uterus is gravid or not. Most of the reticulum tends to lie near the nuclear poles or closely associated with the surface membranes and vesicles. Generally, the tubular structures of the reticulum are directed parallel to the length of the cell. Those tubules and cisternae that lie in close proximity to the surface membrane are sometimes seen to envelope both individual and groups of vesicles.

The close anatomical relationship between these structures and biochemical analysis of the calcium storage properties of smooth-walled reticulum, suggest a role for the vesicles similar to that of the striated muscle t-tubule in the release of calcium ions from the reticulum during the contraction process. Conversely, the rough endoplasmic reticulum is involved in the formation of collagen and elastin as well as in the renewal of the contractile proteins of the myocyte. Ross and Klebanoff (1971) and Ross (1971) demonstrated, using radio-labelled proline, the ability of the myocyte to take up this amino acid and subsequently synthesize and secrete the proteins associated with the collagen matrix and elastic fibres. Using

electron microscope radioautographs, they confirmed that the organelles involved in this process were predominantly the rough sarcoplasmic reticulum, the golgi complex and the surface vesicles.

Along the length of the plasmalemma are multiple surface vesicles that open outwards and measure approximately 60nm in diameter. They alternate with the dense bodies that lie close to or adhere with the cell membrane. Although originally thought to be pinocytotic in nature, most of these vesicles open onto the surface and those that do not are usually found within close proximity to the membrane and not within the more central regions of the cell. Those vesicles that apparently do not communicate with the membrane surface are most likely a result of a sectioning artifact rather than an isolated vesicle. Furthermore these structures are not true vesicles opening onto the surface but rather, as can be seen from their trilaminar membrane, they are flask-shaped invaginations of the plasmalemma. As already mentioned, these vesicles are functionally analogous to the t-tubule system and thus play a role in the movement of ionized calcium across the membrane. Kao (1977) also postulated that they may also provide active transport for proteins and ions, such as sodium and potassium, during the generation of action potentials.

The mitochondria of myocytes are typically small, ovoid and bounded by a double membrane. Fractionation studies carried out by Batra (1972, 1973) indicate that the smooth muscle mitochondria have an extensive calcium binding capacity and thus a possible role in the control of the uterine contraction-relaxation mechanism.

The golgi apparatus is usually distinct and located at one of the nuclear poles. Structurally, it is composed of three to fifteen flat bowed cysternae closely apposed to each other. The cysternae tend to be centrally compressed and peripherally dilated. Other than a role in the production of the stromal components, its function in the myocyte has not been discerned. Centrioles are rarely seen within the smooth muscle cell. When present, they lie close to the nucleus but are not an indication of cell division.

Scattered throughout the cell are numerous free ribosomes and glycogen granules. Electron microscopically they are difficult to distinguish from one another unless histochemical analyses are conducted. Glycogen granules are however, usually larger than the ribosomes.

Each myocyte is bounded by a typical trilaminar membrane of about 15nm thick. Mark (1956) described gaps in the sarcolemma and suggested that these may be artifactual or indicate a partial syncitial nature of the myometrium. His claims have not been substantiated further. External to the sarcolemma is a well defined but often interrupted basal lamina (Ferenczy 1979).

Intercellular connections are seen as closely opposed plasma membranes (Lane & Rhodin 1964) both in the presence and absence of juxtaposed plasma membrane bodies. Gap junctions (Uehara & Burnstock 1970), as these appositions are termed, are seen to be between 2 and 5nm wide. Kao (1977) has surmised that they play a role in the transmission of impulses between the muscle cells.

The myocytes, which are embedded in an abundant connective tissue stroma, are innervated by branches of the autonomic nervous system which form close contacts with the plasma membranes of the cells (Morizaki *et al* 1989). At these points of close contact the nerve fibres contain multiple membrane-bound neuro-vesicles, the contents of which are identifiable in the micropinocytotic vesicles of adjacent cells. Direct axonal penetration into the muscle cell has not been described (Lane & Rhodin 1964, Friederici & DeCloux 1968).

CHAPTER TWO

LEIOMYOMATA: EPIDEMIOLOGY AND AETIOLOGY

1) TERMINOLOGY

1.1) *Historical Perspectives*

The earliest recorded description of uterine leiomyomata is possibly that provided by 'The Father of Medicine' Hippocrates. He listed a number of cases of 'uterine stones' that were delivered of elderly maidens and in all likelihood these phenomena were merely displaced calcareous submucous fibroids. Galen, in the 2nd century CE, described leiomyomata as hard tumours that arose from any portion of the uterus and thus termed them scleroma. He discussed the possibility of their forming in response to chronic inflammation.

During the 11th century CE an Arabo-Persian physician, Avicenna, related the presence of these solid tumours to a myriad of clinical symptoms. These included urinary obstruction, chronic weight loss and pedal oedema. It was not until the late 16th century and early 17th century that the nature of leiomyomata was investigated in any depth. Physicians such as Louis, Meckel and Ryan all provided varying descriptions of these tumours. Ryan, in 1841, incorrectly detailed the structure of leiomyomata as stones of "animal substance, combining the salts of soda, potash and lime, that tend to arise in much the same manner as biliary and urinary calculi" (Mahfouz & Magdi 1941).

In early 1818 Meckel provided the first accurate description of these tumours in his handbook on anatomical pathology. Vogel, in 1843, was the first researcher to document the similarity in composition between uterine myometrium and leiomyomata. His work was later followed by that of Hyenne in 1898, Blanc in 1900 and Guibè in 1901. The origin of the term 'fibroid' was attributed to Rokitansky by Graves while Virchow adopted the term 'myoma laevicellulare', later shortened to 'myoma' (Mahfouz & Magdi 1941).

1.2) *Modern Terminology*

Within current literature there are multiple acceptable synonyms denoting the leiomyomata of uterine tissue. These include *fibroma*, *myoma*, *fibromyoma*, *fibroid* and *leiomyofibroma* (Gompel & Silverberg 1994). Of all of these terms, *fibromyoma* best reflects the character of these benign tumours. That is; predominantly smooth muscle with varying amounts of intermingling fibrous tissue. Even though leiomyomata do not arise from the fibrous components of the uterus, the term *fibroid* is the most common colloquial expression used for these tumours and is firmly entrenched in the literature due to long-term usage (Novak & Woodruff 1974).

2) EPIDEMIOLOGY

2.1) *Age Incidence*

Although there are numerous references to the age incidence of leiomyomata, there is a dearth of actual detailed epidemiological analyses of this topic. However, from the available clinical data, it is apparent that the development of leiomyomata tends to be limited to the reproductive years. The youngest documented case of symptomatic leiomyomata was that recorded in a thirteen year old girl, who presented with an 18 week pelvic mass, menorrhagia and concomitant anaemia (Wisot *et al* 1969, Norris & Zaloudek 1982). Both Novak and Woodruff (1974) and Barber and Graber (1973) quote a report of a leiomyoma found in an 11 year old but this remains unconfirmed. It is apparent that the occurance of these tumours in females below the age of 18 years (Norris & Zaloudek 1982) is extremely rare, with only 9 cases being documented in a study of 1000 uteri (Mafouz & Magdi 1941).

After the age of 20 years, the incidence of leiomyomata, within autopsy specimens, rises to approximately 17.5% (Haines & Taylor 1975). Norris and Zaloudek (1982), Novak and Woodruff (1974) and Vollenhoven *et al* (1990) all quote an incidence figure of 20 to 30% for women of 30 years and older. At 35 years of age 25% of all women can expect to harbour leiomyomata (Miller & Ludovici 1955) and by the time they reach 45 years this figure increases to 40% (Gompel & Silverberg 1994). Leiomyomata are therefore most common in middle-aged women (Norris & Zaloudek 1982) with over 50% of leiomyomata occuring

between the ages of 40 and 50 (Torpin *et al* 1942). Tiltman (1980) in a study of 1000 surgically resected uteri records an overall incidence for leiomyomata of 56,4% for women with an age range of 13 to 85 years.

Finally, there are conflicting reports in the literature as to the regression of these tumours with advancing age. Although Norris and Zaloudek (1982) report that they cease to grow after the cessation of menses, Zeit (1949) demonstrates, in a study of 617 post menopausal women, that leiomyomata greater in size than a 12 week uterus "tend to be troublesome" and indicates surgery for such women.

2.2) ***Population Incidence***

In general it appears as if leiomyomata occur with greater frequency in women of Negroid descent than in those of Caucasian origin (Norris & Zaloudek 1982). Torpin *et al* (1942) studied the incidence of leiomyomata in negro women residing in the neighbourhood of Augusta, Georgia. Of 1741 surgically resected uteri, collected over a 20 year period, the relative frequency of the tumours was consistently 3x greater in the negro women than that occuring in their white counterparts. These figures agree with those quoted by Haines and Taylor (1975) who note an incidence, for a female population over the age of 20 years, of 33% in negroes and 10% in caucasians. It has been estimated that one eighth of all patients seen within gynaecological practice in both Great Britain and the United States of America suffer from fibroids (Haines & Taylor 1975). A similar frequency is noted in Egyptian women, with over 8% of gynaecological admissions as a result of leiomyomata (Mahfouz & Magdi 1941).

2.3) ***Morbidity***

Leiomyomata, although benign, are not always innocuous and remain a significant factor in gynaecological morbidity. It is estimated that 60% of all laparotomies performed on women for pelvic disease are directly related to the presence of fibroids (Miller & Ludovici 1955). In Australia upwards of 40% of hysterectomies performed are due to symptomatic leiomyomata. This produces a massive $100 million public health cost bill per annum, due to the burgeoning costs of outpatient attendances, failed medical therapy, hospitalisation and surgery for these patients (Vollenhoven *et al* 1990).

2.4) *Risk Factors*

According to Ross *et al* (1986) all of the risk factors for leiomyomata are underpinned by exposure to oestrogen in the absence of progesterone. They demonstrated in a long term study involving over 1000 women, that the risk of developing fibroids decreased by 31% in those women who had used oral contraceptives for 10 years or longer and that the higher the dose of progestin in the oral contraceptive, the greater the protection offered. The results of this study have been criticized in terms of the composition of the sample with a possible indication bias (Ratner 1986). Parazzini *et al* (1992) was unable to demonstrate any significant relationship between oral contraceptive use and fibroid risk.

Fibroid risk also appears to decrease with each subsequent term pregnancy and those women with a history of five term pregnancies are at one quarter the risk of developing fibroids when compared to nulliparous women (Ross *et al* 1986). Increasing weight has been demonstrated to be a significant risk factor with a 21% increase in risk for each 10Kg gained over a 55Kg base line. The smoking of 20 cigarettes per day reduces the chances of fibroids to two thirds that of non-smokers. Factors that have been shown to have no relationship to the development of these tumours are the presence of intrauterine devices, a history of spontaneous abortions and recurrent urinary tract infections (Parazzini *et al* 1988).

3) AETIOLOGY

Despite their commonality, the aetiology of leiomyomata remains largely unknown. Most of the investigations carried out in the hope of eliciting the causative agent(s) may be placed in one of two categories. These are:

* The Hormonal Theories of Causation
* The Genetic and Cytogenetic Theories

3.1) *The Hormonal Theories of Causation*

Although many hormones have been suggested as aetiological factors in the pathogenesis of leiomyomata, oestrogen is the most commonly implicated of the reproductive steroid hormones. The origin of the oestrogenic theories of causation is twofold. Firstly, the increased risk of tumour development has been linked to the presence of continuous unchallenged oestrogen flow (Lipschütz 1942, Ross *et al* 1986). Secondly, as it is generally accepted that leiomyomata are tumours of the

reproductive years (Novak & Woodruff 1974); forming during the period of gonadal activity and usually regressing after menopause (Gompel & Silverberg 1994), the basic assumption is made that they must be related to the reproductive steroid hormones.

A fair amount of clinical evidence supports continuous oestrogenic stimulation as being a major aetiological factor in leiomyoma development. Vollenhoven *et al* (1990) note an association between nulliparity and fibroids with the risk of tumour development decreasing as uninterrupted oestradiol production is altered during the state of pregnancy. In a few isolated cases, massive leiomyomatous growth has been reported following the administration of clomiphene (Felmingham & Corcoran 1975). It is hypothesized that clomiphene-induced follicular development (Frankel & Benjamin 1973) causes rising oestrogen output leading to increased fibroid formation. Similar to this, increases in the dimensions of leiomyomata have been reported in patients taking oestrogen preparations (Prakash & Scully 1964), progestins and other drugs with oestrogenic potential (Mixson & Hammond 1961). In opposition, endometrial hyperplasia, as evidence of prolonged unopposed or excessive oestrogen stimulation occurs in under 10% of fibroid uteri and thus does not justify the conclusion that continuous oestrogen supply is the definitive cause of leiomyomata (Henderson 1941). Several attempts have also been made to explain the high incidence of fibroids in Negroes on the basis of long-standing elevations in oestrogen, due to ovarian cystic change in response to chronic pelvic inflammatory disease, however the ovaries of most of these patients have been found to be structurally normal (Miller & Ludovici 1955, Torpin *et al* 1942).

Biochemically the evidence related to the oestrogenic hypothesis is conflicting. When leiomyomata are compared to normal myometrium, most researchers note no difference or only slightly higher concentrations of oestradiol receptors (Farber *et al* 1972, Pollow *et al* 1978, Tamaya *et al* 1979). There are few reports of significant increases in the oestrogen receptor content of leiomyomatous tissue (Wilson *et al* 1980). Oestrogenic theory may also be discounted as the serum oestradiol level in patients, with fibroids, is similar to that of a normal control group (Spellacy *et al* 1972). Such fibroids also tend to respond to reproductive steroid hormone cycling in much the same manner as normal myometrium (Soules & McCarty 1982). Some biochemical support for the theory exists in the form of the lower conversion rates, within leiomyomata, for oestradiol to oestrone (Pollow *et al* 1978), suggesting an increase in the intra-tumour oestradiol concentration.

This hypothesis is further substantiated by Deligdish and Loewenthal (1970) who note the constant presence of endometrial glandular hyperplasia at the margins of submucous fibroids, which in itself may be indicative of a locally hyper-oestrogenic environment.

Experimental attempts to elicit leiomyomata in response to excessive oestrogen are at best confusing. Nelson (1937, 1939), after injecting guinea pigs with oestrogenic hormones, demonstrated the presence of endometrial hyperplasia as well as growths of the outer longitudinal muscle layer. These growths, although initially classified as leiomyomata, were predominantly fibrous tissue and histologically dissimilar to human fibroids. The tumourigenic potential of continuous high dose oestrogen administration was shown in the experiments of Lipschütz (1942) in which multiple tumours of the uterus, spleen, pancreas and abdominal wall were produced. He noted that the abdominal tumours commonly occurred in the absence of a uterine counterpart and were structurally different to classical leiomyomata. He thus concluded that although excessive oestrogen has tumourigenic potential it does not necessarily initiate the formation of uterine fibroids. He also noted that the pathogenic potency of unchallenged oestrogen dissipated in the presence of a progesterone or testosterone challenge.

Although it cannot be proven that leiomyomata are the direct result of the presence of excessive oestrogen there is sufficient evidence to confirm that they are at least partially hormonally dependant and that increases in oestrogen stimulus, serum growth hormone or human placental lactogen (HPL) will encourage increased tumour growth (Buttram & Reiter 1981). The administration of some oral contraceptives in particular the progestins, have been implicated in both enlargement and the development of cellular atypia in fibroid tumours occurring in the gravid uterus (Fechner 1968, Norris *et al* 1988). Further evidence of the hormonal dependence of these tumours is found in studies where increased mitotic activity is present during the secretory phase of the menstrual cycle (Kawaguchi *et al* 1989). Similar findings have been recorded in the fibroids of women receiving progesterone preparations (Tiltman 1985). Also, cell cultures of fibroid tissue that have had their medium enriched with oestrogen and progesterone are ultrastructurally more differentiated than their untreated counterparts, as evidenced by mature myofilament ultrastructure and increased dense body numbers (Kawaguchi *et al* 1985).

A number of polypeptide factors that stimulate cell proliferation by binding to specific cell membrane receptors may play a mediatory role in the growth of fibroids. These include epidermal growth factor (EGF)(Hofmann *et al* 1984) and transforming growth factor alpha (TGF-α)(Goustin *et al* 1986). In women rendered hypo-oestrogenic following lutenizing hormone releasing hormone (LHRH) agonist treatment, the binding of EGF to the cell membranes of fibroids is decreased while that of normal myometrium remains constant (Lumsden *et al* 1988), suggesting that the role of oestrogen may be partially controlled by the action of EGF.

The assumption that the regression of fibroids is purely consequential to the withdrawal of an oestrogenic stimulus, both post-menopausally and subsequent to gonadotropin releasing hormone (GnRH) agonist therapy (Friedman *et al* 1989, Letterie *et al* 1989), is possibly erroneous. Post-climacteric decreases in the dimensions of leiomyomata may perhaps be attributable to a reduction in the blood supply of the tumours due to the normal degeneration of the uterine arteries. This degeneration takes the form of intimal proliferation, fibrosis and medial calcification (Hendrickson & Kempson 1992). In much the same manner, involution of leiomyomata may be produced by radiation therapy with the induction of a uterine vascular endarteritis (Montgomery & Long 1958).

3.2) *The Genetic and Cytogenetic Theories*

Heredity does not appear to be a significant factor in the aetiology of leiomyomata and even though there appears to be some racial difference in the incidence of the tumour, its overall high frequency makes the establishment of a familial basis of inheritance most difficult.

Although cytogenetic analyses of leiomyomata fail to reveal a specific defect that may be responsible for the formation of the tumours, upwards of 30% of leiomyomata will demonstrate some form of clonal chromosomal abnormality (Hu & Surti 1991). Due to the myriad of abnormalities that do occur, including deletions, insertions and complex translocations, it has been suggested that the formation of leiomyomata may follow any one of a number of possible different cytogenetic pathways (Hu & Surti 1991, Meloni *et al* 1992).

CHAPTER THREE

PATHOLOGY OF LEIOMYOMATA

1) LOCATION

Leiomyomata are more commonly located in the corpus of the uterus than in the cervix. Within the uterus, tumours may be submucous, intramural or subserous in position (Figure 6).

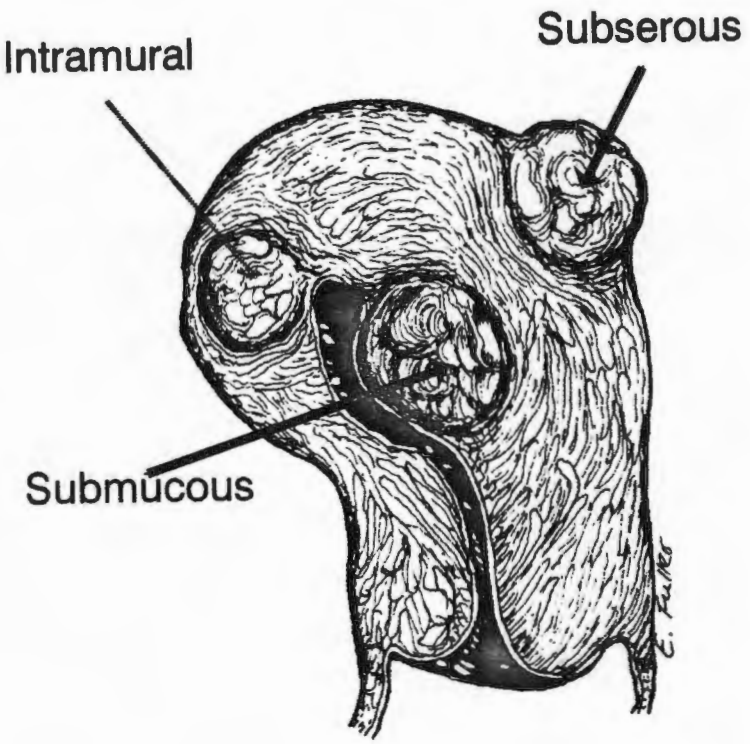

Figure 6: Schematic representation of a sagital section through the uterus demonstrating the location of leiomyomata. Note the distortion of the uterine cavity by the submucous leiomyoma.

1.1) *Submucous fibroids*

Only 5% of uterine leiomyomata are located submucosally (Novak & Woodruff 1974). These tumours tend to be solitary and usually remain sessile at their myometrial attachment but may become pedunculated, occasionally prolapsing into the cervical orifice of the vagina. Tumour detachment infrequently occurs subsequent to pedicle torsion and the free tumour may be passed per vaginum. Due to their position, submucous fibroids are prone to inflammation, ulceration and necrosis (Haines & Taylor 1975).

1.2) *Intramural fibroids*

Up to 70% of all uterine leiomyomata are intramurally situated (Llewelyn-Jones 1982), with most occuring within the posterior uterine wall. When large, these tumours bulge under the mucosa or serosa distorting the uterine cavity or serosal surface respectively. Diffuse leiomyomatosis, a rare clinicaly benign disorder (Clement & Young 1987), is characterised by the presence of numerous small confluent leiomyomatous nodules that replace the myometrium (Murray & Glynn 1924). The larger nodules are partially delimited by thin walled vascular spaces. The uterus is usually symmetrically enlarged and widespread involvement of the myometrium precludes myomectomy (Lapan & Soloman 1979).

1.3) *Subserous fibroids*

Subserous tumours are usually multiple, herniating beneath the peritoneal serosa and distorting the surface of the uterus (Haines & Taylor 1975). These frequently pedunculated tumours may adhere to other intra-abdominal organs from which they derive a second blood supply - so called "parasitic" myomata (Novak & Woodruff 1974).

1.4) *Cervical fibroids*

Usually solitary, cervical leiomyomata tend to be located in the posterior cervical wall (Haines & Taylor 1975). A true cervical fibroid is completely surrounded by the wall of the cervix while a paracervical fibroid is normally embedded in the outer wall of the cervix from which it projects (Haines & Taylor 1975).

1.5) *Intraligamentary fibroids*

These are usually outgrowths of a fibroid between the layers of the broad ligament but may be derived *in situ* from the smooth muscle of the ligament. When large they may burrow outward forming retroperitoneal masses (Haines & Taylor 1975).

2) MACROSCOPIC APPEARANCE

When confined to the uterus, leiomyomata are typically spherical but may assume any shape when protruding from the uterine surface (Figure 7). They are well demarcated tumours with a distinct line of cleavage at their periphery. When the uterus is cut, retraction of the surrounding myometrium results in the tumours standing proud so that they are usually easily shelled out.

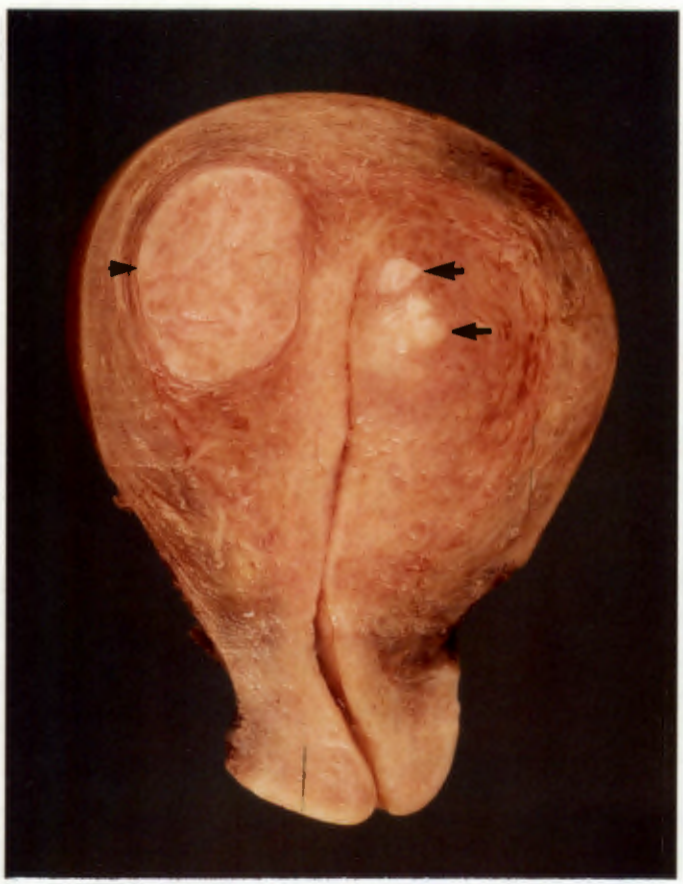

Figure 7: Photograph of a sagital section through a uterus. A clearly circumscribed intramural leiomyoma is seen on the left (arrowhead) and small seedling leiomyomata are visible on the right (arrows). Scale 3:5.

Fibroids are rarely solitary (Loeffler & Noble 1970, Malone 1969) with up to 225 being counted in a single uterus (Lapan & Soloman 1979). Where more than one tumour occurs, they usually vary in size and may easily attain a diameter of 20cm and a weight of up to 10Kg.

Despite there being a number of histological subtypes of leiomyomata their gross appearance is similar. Each individual leiomyoma is surrounded by a psuedocapsule formed by a zone of compressed muscle beyond the tumour. A small amount of aerolar tissue intervenes. In sharp contrast to the fleshy dark red of fresh unfixed myometrium, leiomyomata are firm, pearly white to tan in colour with a whorled trabecular pattern reminiscent of watered silk (Llewelyn-Jones 1982). The macroscopic appearance of fibroids is altered by infarction, haemorrhage, calcification and necrosis, especially when the uterus is in the gravid state or following high dose progestin therapy (Goldzieher *et al* 1966).

3) HISTOPATHOLOGY

3.1) *Light microscopic appearance*

Histologically, leiomyomata are identified by their nodular circumscription and characteristic anastomosing whorled fascicles of fusiform cells of relatively uniform size that are out of phase with the surrounding myometrium. Although leiomyomata appear to be more cellular than the neighbouring myometrium, mitotic figures are rare, with few tumours having more than 5 mitoses per 100 high power fields (Tiltman 1985). Nuclear atypica is uncommon and most nuclei are usually small and elongated with blunt tapered ends and a finely dispersed chromatin network. Palisading of nuclei into a pattern suggestive of a nerve sheath tumour is occasionally seen. Ultrastructurally these tumours are merely leiomyomata with an unusual nuclear arrangement (Gisser & Young 1977, Janovski & Taki 1962) that may reflect a metabolic rather than a neural histogenetic abnormality (Abenoza & Sibley 1987).

Each individual fibroid consists of bundles of smooth muscle fibres with abundant eosinophilic, fibrillar cytoplasm separated from each other by a connective tissue stroma of varying quantity (Figure 8). Mast cells are located in capillaries, vessel walls and the pseudocapsule (Fox & Abell 1965). Special stains, usually trichromic, are required to differentiate the varying tissue components.

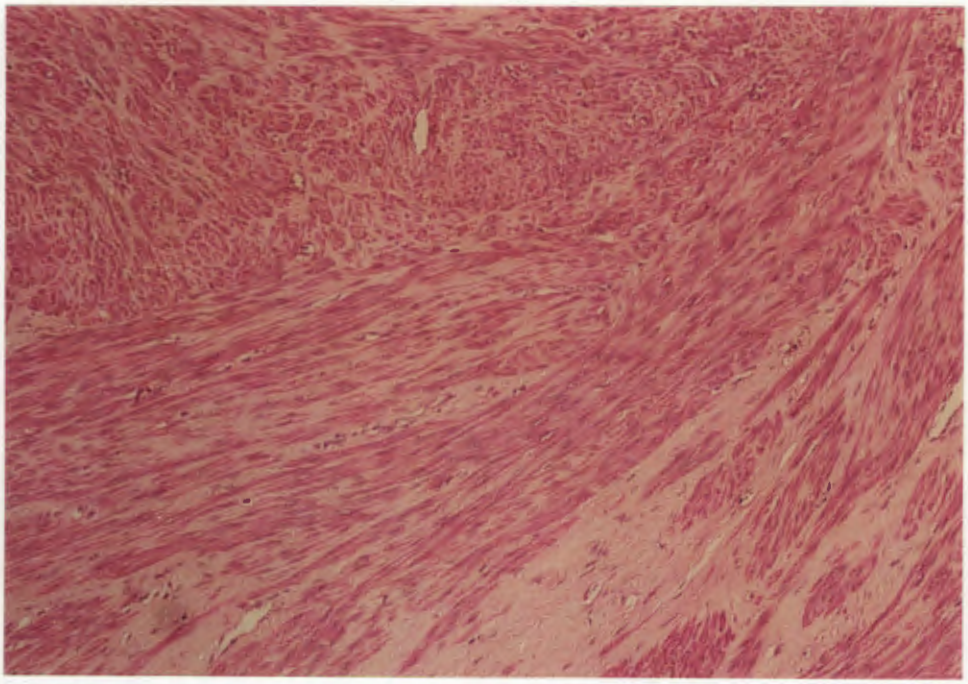

Figure 8: Light micrograph of a H&E stained section of an intramural fibroid. Note the bundles of smooth muscle fibres and intervening connective tissue. Print magnification x110.

3.2) *Ultrastructure*

Ultrastructurally the cells of the leiomyomata are difficult to distinguish from those of normal myometrium (Ferenczy 1979). Nuclei are invaginated with uniform, marginally condensed chromatin and contain nucleoli of regular outline. Organelles are sparse and perinuclear. Abundant myofilaments, multiple peripheral dense bodies, prominent pinocytotic vesicles and a well defined basement membrane are characteristic. Leiomyomata differ ultrastructurally from normal myometrium in that they have increased numbers of mitochondria and free ribosomes as well as fractionally larger nuclei (Ferenczy & Richart 1974, Ferenczy *et al* 1971).

3.3) *Vascular supply*

As leiomyomata commonly undergo degeneration, it is generally accepted that the growth of the tumour outstrips the available blood supply, however, there are few detailed studies of the vascular supply of leiomyomata that substantiate this

claim. Faulkner (1944), using injection methods, demonstrated great variability in the vascularity of myomata where no tumour had a single nutrient artery but rather a number of small vessels that penetrated the pseudocapsule. Radioangiography (Farrer-Brown *et al* 1970b) has confirmed that the tumours have no regular intrinsic vascular pattern, are less vascular than the surrounding myometrium and that venous congestion is probably a consequence of localised obstruction.

4) HISTOLOGICAL SUBTYPES

There are four main histological variants of uterine leiomyomata. These include:

* Cellular leiomyomata
* Epithelioid leiomyomata
* Atypical leiomyomata
* Lipoleiomyomata

4.1) *Cellular leiomyomata*

Characteristically, cellular leiomyomata consist of small, densely packed, spindle shaped cells with little intervening collagen. The parallel arrangement of reticular fibres in relation to the fascicles of cells and the lack of a prominent vascular bed, distinguish them from endometrial stromal tumours (Baggish & Woodruff 1972, Tavassoli & Norris 1981). Although definitely more cellular than the surrounding myometrium, less than 5 mitotic figures per 10 high power fields are usually present (Burns *et al* 1979, Christopherson *et al* 1972). The elongated nuclei are of bland appearance and little to no cellular atypia exists (Kempson & Bari 1970, Saksela *et al* 1974) although occasional palisading of nuclei has been described. Cellular leiomyomata display non-malignant behaviour and have an excellent prognosis (Burns *et al* 1979).

4.2) *Epithelioid leiomyomata*

Epithelioid leiomyomata are classified as leiomyomata in which the fibres have undergone modification so that they resemble epithelial elements (Kurman & Norris 1976). Three main categories of epithelioid tumours are recognised, all of which behave in a benign manner. They are:

* Clear cell leiomyomata
* Leiomyoblastoma
* Plexiform leiomyomata

Clear cell leiomyomata are distinguished by their polygonal cells with abundant, clear, vacuolated cytoplasm and a well defined basement membrane. The nucleus is often eccentrically displaced resulting in a signet ring appearance. Minimal lipid or mucin are histologically detectable while glycogen is plentiful (Rywlin *et al* 1964). Ultrastructurally the cells resemble those of the smooth muscle components of the myometrium with the exception of large empty mitochondria which may account for the vacuolated appearance (Hyde *et al* 1989, Mazur & Priest 1986).

The cells of leiomyoblastomata may be round or polyhedral with strongly eosinophilic homogenous cytoplasm and voluminous nuclei. The presence of myofilaments and marginal dense bodies confirms their smooth muscle origin (Chang *et al* 1977, Wolfe & Mackles 1963).

Plexiform leiomyomata are small tumours (Goodhue *et al* 1974) that are usually solitary (Budinger & Greene 1964). As a result of their small size and benign nature they are normally only incidental findings. They are identifiable as randomly oriented cords, or plexiform masses, of uniform cells with vesicular rounded nuclei, separated by abundant connective tissue (Evans *et al* 1988). The cells of these tumours have been positively identified both ultrastructurally and immunohistochemically as of smooth muscle origin (Goodhue *et al* 1974, Kaminski & Tavassoli 1984, Nunez-Alonso & Battifora 1979).

4.3) *Atypical leiomyomata*

Synonyms for atypical leiomyomata include bizarre, symplastic and pleomorphic leiomyomata. They usually occur during the fourth decade of life (Burns *et al* 1979) and are difficult to distinguish from sarcomata due to their similar histological features (Hart & Billman 1978, Montague *et al* 1965, Przybora 1961, Symmonds & Dockerty 1955). These tumours consist of numerous mono- or multinucleated atypical cells that are either distributed throughout the fibroid or contained within specific foci. The nuclei are usually enlarged, hyperchromatic and show prominent chromatin clumping. Large intranuclear inclusions of cytoplasm are

common. These tumours are distinguishable from leiomyosarcomata by their lack of mitotic figures (Gompel & Silverberg 1994).

4.4) *Lipoleiomyomata*

Lipoleiomyomata are rare tumours usually encountered as incidental findings in patients over 40 years of age (Jacobs *et al* 1965). Fat deposition may be either diffuse or occur in localized areas within the leiomyoma. Microscopically these tumours consist of adult type adipose tissue divided into lobules by thin connective tissue septae. Varying amounts of smooth muscle cells are interspersed between the lobules of fat (Pounder 1982). The histogenesis of these tumours remains unclear. It has been suggested that they may be derived from embryonic nests of mesodermal cells (Demopoulos *et al* 1973) or by metamorphosis of uterine smooth muscle (Willèn *et al* 1978). Sieinski (1989) postulated that lipomatous differentiation of immature neoplastic cells may occur within pre-existing leiomyomata simultaneously with leiomyomatous differentiation.

5) HISTOGENESIS

Although the histogenesis of leiomyomata is unknown, two main schools of thought predominate. Schwarz and Wissner (1949) suggested that the intimate anatomical relationship between uterine blood vessels and minute myomata was indicative of the tumours having arisen from the smooth muscle components of the vessels. In support of the vascular theory, Honoré (1977, 1978) identified a leiomyoma with haemangiopericytomous foci and implicated the vascular pericyte as the mother cell of both. He suggested that the tumour arose from a single cell type, possibly an undifferentiated adventitial fibroblast, with the potential for differentiation depending on local and/or systemic factors.

Using tissue culture techniques, the differences between the cells of leiomyomata and fibroblasts led Miller and Ludovici (1955) to conclude that fibroids develop from uterine smooth muscle. Even though there are a number of histological subtypes of leiomyomata, they are ultrastructurally similar, suggesting a common myometrial origin. The varied morphological patterns possibly reflect the capacity of uterine smooth muscle to undergo a wide spectrum of alteration (Mazur & Kraus 1980). Cramer and Patel (1992) have postulated that the non-random distribution of leiomyomata, where the smooth muscle cells of the corpus uteri are

at greater risk for neoplastic transformation than those of the cervix, perhaps supplies a clue to the histogenesis of fibroids.

The unicellular origin of leiomyomata is unquestionable. Townsend *et al* (1970) demonstrated that each cell within a leiomyoma is of identical glucose-6-phosphate dehydrogenase type, although the type may vary between tumours within the same uterus. Their results suggest that mutation rather than environmental factors may result in the formation of fibroids.

6) DEGENERATIVE CHANGES

Standard leiomyomata are commonly altered, both macroscopically and microscopically, by secondary degenerative changes. Such changes typically include; hyaline degeneration, cystic degeneration, calcification, fatty degeneration and necrosis.

6.1) *Hyaline degeneration*

This type of degeneration occurs to a varying degree in almost all leiomyomata and accounts for over 60% of the forms of degeneration seen in these tumours (Persaud & Arjoon 1970). As the connective tissue elements of leiomyomata appear to be the most extensively affected, hyaline degeneration occurs to a greater degree in the less cellular forms of the tumour. Macroscopically the degenerative areas appear pale to translucent, are of homogenous character and may be scattered throughout the tumour or appear as large interlacing bundles. Histological examination reveals the affected areas to be highly eosinophilic with a marked loss of cellularity and an absence of the normal architectural pattern.

6.2) *Cystic degeneration*

Progressive hyaline degeneration tends towards liquefaction with the formation of multiple small irregular cystic spaces that progressively become filled with a colourless or blood stained gelatinous material. The cysts may enlarge sufficiently so as to obliterate the majority of the tumour tissue. Only 4% of tumours with secondary changes demonstrate cystic degeneration (Persaud & Arjoon 1970). On gross anatomical examination the affected tumours are variegated due to the ragged cystic cavities with only small remnants of intact tumour tissue

intervening. When examined microscopically, the cystic spaces lack an epithelial lining.

6.3) *Calcification*

The calcification of leiomyomata is an infrequent event that occurs following liquefaction, haemorrhage or necrosis. It is also seen subsequent to the circulatory impairment that accompanies the climacteric as well as within subserosal leiomyomata with small pedicles. Calcium is deposited in the carbonate and phosphate forms as either small scattered granular areas or as extensive stony masses. When stained with haematoxylin, calcium deposits are identifiable as amorphous lakes of variable dimensions and shapes.

6.4) *Fatty degeneration*

Fatty change, usually associated with antecedent degeneration, is extremely difficult to distinguish from uterine lipomata and is rare, forming only 3% of secondary changes in leiomyomata (Persaud & Arjoon 1970). The typical whorled pattern is obliterated in the affected areas and replaced by small vacuolated lipid containing cells that lend such areas a soft homogenous character.

6.5) *Necrosis*

Necrosis of leiomyomata commonly occurs secondarily to impairment of the tumour blood supply and may be diffuse or focal. Recent necrosis is identifiable as haemorrhagic areas (Briscoe 1964) while long standing necrosis usually adopts a greenish-grey appearance. Red degeneration, a rare form of necrosis in which the whole or part of a fibroid is necrotic, is usually associated with the gravid uterus. The necrotic areas appear mottled, dark red and not dissimilar to the appearance of raw beef. The whorled appearance of the tumour remains intact and thrombosed vessels are commonly seen at the periphery. Histologically patchy necrosis and muscle fibre haemorrhage are characteristic. The colour of red degeneration may be derived from haemolysis or due to the extravasation of blood following obstruction of the venous return, with the resultant increased back pressure causing venous capillary rupture. Unlike necrosis following infection, red degeneration is supposedly aseptic (Faulkner 1947).

7) ASSOCIATED PATHOLOGY

7.1) *Intravenous Leiomyomatosis*

Intravenous leiomyomatosis may be defined as the intravascular extension of a benign myomatous tumour (Steiner *et al* 1963). The majority of recorded cases have occurred in women in the fourth decade of life with a mean age of 42 years (Clement *et al* 1988). Few cases have been noted in women below the age of 40 (Nogales *et al* 1987). Although usually identified microscopically, when sufficiently large, the tumours may be visible to the naked eye as coiled or nodular growths in the myometrium, with worm-like extensions into the veins of the uterus and broad ligament (Borland & Wotring 1964). The consistency of the tumours ranges from soft and spongy to rubbery and firm. They are often lobulated, with small intervening clefts and are most commonly pinkish-white to grey in colour (Edwards & Peacock 1966). Histologically they are composed of endothelial-coated masses of smooth muscle cells with some fibrous tissue and are located within the lumina of sinusoidal vessels (Norris & Pramley 1975). The venous, rather than lymphatic nature of the vessels is confirmed by the presence of blood and/or thrombi. The classical whorled pattern of leiomyomata is absent in these tumours. Mitotic figures are rare and cellular atypia uncommon (Nogales *et al* 1987). These tumours have a favourable prognosis (Thompson *et al* 1962) due to their benign behaviour, with the only recorded deaths having occurred as a result of inferior vena cava obstruction (Harper & Scully 1961). As with classic leiomyomata, the histogenesis of this bizarre growth variance is unknown. It is possible that they arise within the musculature of the vessels they invade or as vascular extensions of existing leiomyomata (Borland & Wotring 1964). The differential diagnosis of intravenous leiomyomatosis includes vessel invasion of a leiomyosarcoma, 'benign metastasizing leiomyoma' and endometrial stromal sarcoma (Norris & Pramley 1975).

7.2) *Benign Metastasizing Leiomyomatosis*

Within the literature there are a number of reports of uterine leiomyomata that have supposedly undergone metastasis (Abell & Littler 1975, Ariel & Trinidad 1966, Cramer *et al* 1980, Idelson & Davids 1963, Konis & Belsky 1966, Steiner 1939). These metastatic foci, which predominantly occur in the lungs and pelvis, are normally asymptomatic and are thus usually reported as incidental findings (Ariel & Trinidad 1966). When foci produce symptoms, these are usually in

response to venous obstruction (Steiner 1939). Although metastatic foci are generally innocuous, death may occur due to secondary cardiac failure in the presence of severe pulmonary obstruction (Steiner 1939).

From the reported cases it is not possible to discern whether metastatic leiomyomatosis is a true entity or not. The possibility exists that the tumours arise as *de novo* events in the lungs or pelvis. Also, in a number of the reported cases, the discovery of metastatic foci subsequent to surgery for uterine leiomyomata, is suggestive of an iatrogenic event following tumour seeding during surgery (Idelson & Davids 1963). Idelson & Davids (1963) suggest that many of the reported metastatic occurrences may have primarily arisen in undiagnosed sarcomatous tumours rather than in benign leiomyomata. The fact that the mitotic rate of the metastatic tumours is negligible tends to dispel this theory (Cramer *et al* 1980).

7.3) *Leiomyomatosis Peritonealis Disseminata*

Leiomyomatosis peritonealis disseminata is an extremely rare condition characterised by the proliferation of leiomyomata, on the peritoneal surfaces of the pelvic and abdominal cavities (Aterman *et al* 1977), which tends to be limited to women of reproductive age. As this condition is usually an incidental finding at caesarian section, it has been loosely associated with the gravid state (Williams & Pavlick 1980). The vague relationship of leiomyomatosis peritonealis disseminata to pregnancy and the reproductive years has prompted the assumption that the condition is in some way related to endocrine function (Taubert *et al* 1965). The majority of the nodules, which measure only a few millimetres in diameter, often involute following termination of the pregnancy (Aterman *et al* 1977). Histologically the nodules are composed of numerous spindle shaped smooth muscle cells, fibroblasts, decidual cells and collagen fibres (Sutherland *et al* 1980). Ultrastructurally the spindle shaped cells of these nodules are fibroblastic in nature with no intracellular myofilaments and no distinct individual basement membranes (Winn *et al* 1976). There have, however, been reports of ultrastructural sections of nodules demonstrating the typical characteristics of smooth muscle cells (Williams & Pavlick 1980).

It has been suggested that the structural components of the nodules that comprise leiomyomatosis peritonealis disseminata, may arise from any of a number of sources. These include; mature smooth muscle cells, undifferentiated mesenchymal and/or myometrial cells, smooth muscle elements of the media of

blood vessels and finally as the fibrous differentiation of a decidual reaction (Aterman *et al* 1977). In all likelihood this condition arises due to multicentric development of leiomyomata rather than due to metastasis or peritoneal seeding (Nogales *et al* 1978).

7.4) *Sarcomatous Change*

The malignant degeneration of leiomyomata is an uncommon event with a recorded frequency of rarely more than 0.5% (Leibsohn et al 1990, Novak & Anderson 1937, Parker et al 1994, Przybora 1961). Due to the similarity in the signs and symptoms between leiomyomata and leiomyosarcomata, malignant change is rarely diagnosed preoperatively (Novak 1958). Even postoperatively the identification of sarcomatous change within leiomyomata is extremely difficult, as leiomyomata are often large and rarely singular making sampling problematic.

CHAPTER FOUR

LEIOMYOMATA: A CLINICAL OVERVIEW

1) MANIFESTATIONS AND SEQUELE

Although no reliable figures are available, it is estimated that 20 to 50% of all leiomyomata will manifest in one form or another (Babaknia *et al* 1978, Ingersoll 1963). The nature and severity of the symptoms produced by these tumours is largely dependant on their number, size and location.

1.1) *Pelvic Pain*

Pelvic pain, although a relatively common symptom reported by patients who harbour leiomyomata (Table 1), can rarely be attributed specifically to the presence of the tumours and is most often the result of co-incident pelvic disease (Buttram & Reiter 1981). Severe acute pain has been associated with the acute necrosis of fibroids in response to LHRH agonist therapy (Vollenhoven *et al* 1990) as well as with cases of torsion of the pedicle of subserous tumours (Morton 1958).

1.2) *Menorrhagia*

As seen in Table 1, a review of the pertinent literature reveals menorrhagia as the presenting symptom in upwards of 28% of patients with fibroids. Even though clinical anaemia is not consistently present in all the patients, its prevention as well as the associated morbidity and costs of treatment make menorrhagia a symptom of significance.

Although previously mooted, the presence of menorrhagia cannot be attributed solely to the ulceration of the surface of submucous fibroids (Sampson 1913) as firstly; such fibroids occur with a frequency of less than 5% (Novak & Woodruff 1974) and secondly most patients with menorrhagia usually harbour intramural or subserous fibroids (Rubin & Ford 1974).

Miller and Ludovici (1955) suggest that anovulation occurs with greater frequency in leiomyomatous women and may possibly be responsible for the development of menorrhagia. However, as most of these women demonstrate regular cycling, the diagnosis of dysfunctional bleeding may largely be precluded.

Normal menstruation has been attributed, by some, to the contractility of the myometrium. The presence of leiomyomata may thus interfere with the normal contracting power of the uterus and so produce abnormal bleeding (Faulkner 1944). The most likely cause of menorrhagia is the compression of the arcuate and/or radial veins of the intramural and subserosal myometrium by an adjacent tumour mass. This produces congestion and dilatation of the endometrial venous plexi and may result in menorrhagia (Farrer-Brown *et al* 1970b, 1971).

Table 1: Symptoms of leiomyomata.

Year	Reference	*N*	Pelvic pain	Menorrhagia	Infertility
1933	Miller & Tyrone	94	49	57	69
1938	Counseller & Bedard	523	128	137	196
1950	Finn & Muller	430	173	98	52
1951	Munnell & Martin	236	56	41	30
1954	Newman	755	28	354	71
1958	McCormick	66	48	18	20
1967	Brown *et al*	95	28	37	14
1970	Loeffler & Noble	144	46	88	42
1970	Persaud & Arjoon	298	223	229	-
1974	Rubin & Ford	100	63	53	13
1979	Ranney & Frederick	51	34	13	25
Total		2792	876 (31.3%)	771 (27.6%)	532 (21.3%)

1.3) *Infertility*

A fairly high percentage of women with symptomatic leiomyomata complain of infertility (Table 1). Of these, very few cases can be directly linked to the presence of the tumours and most often the cause of infertility is related to

conditions such as pelvic inflammatory disease and endometriosis (Rubin & Ford 1974). The mechanism by which leiomyomata cause infertility, in those cases where no other cause can be established, is controversial. Miller and Ludovici (1955) explained infertility on the basis of anovulation associated with fibroids. This theory has not had much support as most of the patients have normal cycles.

Prostaglandins in seminal fluid facilitate the transport of sperm by causing rhythmic uterine contractions during intercourse. Disruption of this mechanism by fibroids may prevent sperm from reaching the ovum (Coutinho & Maia 1971). Likewise, severe distortion of the uterine cavity increases the distance which sperm have to travel and so reduces the chances of fertilisation (Hunt & Wallach 1974). The most likely reason for infertility, due to leiomyomata, is mechanical obstruction due to tumours in or near the tubal orifices (Chalmers 1948, Roberts & Marshall 1961).

Supposed infertility may also be due to unsuccessful nidation of the fertilised ovum in those areas where the endometrium is flattened or ulcerated due to an underlying submucous fibroid (Hunt & Wallach 1974, Rubin 1958, Stevenson 1964). Venous dilatation and congestion due to fibroid pressure may also sufficiently alter the endometrial environment so as to prevent implantation (Farrer-Brown *et al* 1970b, 1971).

1.4) ***Impingement/Obstruction***

Anteriorly growing tumours, particularly those of the cervix, occasionally exert sufficient pressure on the bladder so as to produce symptoms of urinary retention, stress incontinence and suprapubic discomfort (Radman 1961). Upper urinary tract dilation, usually manifesting as hydronephrosis, has been identified as an obstruction effect in association with massive fibroids (Everett 1958). The condition usually resolves, in the absence of pelvic inflammatory disease and urinary tract infection, following removal of the offending fibroid (Weiss *et al* 1975). Fibroid tumours that extend posteriorly from the uterine cervix or body may impinge on the rectosigmoidal colon producing constipation and/or frank intestinal obstruction (Radman 1961).

1.5) *Ascites and Spontaneous Haemoperitoneum*

Pedunculated subserosal and parasitic leiomyomata sporadically undergo torsion. The resultant vascular congestion and oedema produces a fluid exudate clinically seen as ascites. Alternatively, ascites may also result from peritoneal irritation due to large subserosal fibroids (Williamson *et al* 1972). Ascitic fluid may be transferred, via the transdiaphragmatic lymph channels, to within the pleural cavity, to present as a hydrothorax (Gianoutsos & Laverty 1975).

Spontaneous haemoperitoneum is a rare but potentially life-threatening complication of leiomyomata (Buttery 1972). Persistent massive intraperitoneal haemorrhage occurs when the enlarged veins coursing over the surface of subserous fibroid rupture (Saidi *et al* 1961). A number of mechanisms may cause vessel disruption. These include excessive stretch on vessels due to a rapidly growing subserosal fibroid, engorgement of pelvic organs during menstruation, as well as tearing of any peritoneal adhesions related to a parasitic fibroid following trauma or abdominal straining (McNeil 1952).

1.6) *Polycythemia*

A well established although uncommon complication of fibroids is the inappropriate production of erythropoietin (Ep) leading to an erythrocytosis. Weiss *et al* (1975) suggest that back pressure on the renal parenchyma, due to fibroid-induced obstruction, produces a state of relative tissue hypoxia with a subsequent increase in Ep production. However, the fact that a number of leiomyomata from polycythaemic patients, with elevated serum Ep levels, demonstrate high levels of Ep activity (Weiss *et al* 1975) is suggestive that some leiomyomata produce Ep (Wrigley *et al* 1971) in the absence of a negative feedback mechanism (Ossias *et al* 1973). Following removal of the tumours serum Ep levels usually return to normal and the accompanying clinical symptoms abate (Payne *et al* 1969, Nedwich *et al* 1962, Thomson & Marson 1953).

1.7) *Complications of Pregnancy and Partuition*

Leiomyomata are becoming an increasingly important feature in pregnancy as many modern women are delaying childbearing until their late thirties, coinciding with the time of increased risk of fibroid development. Even so only 10% of women with fibroids will experience complications related to these tumours during the

course of their pregnancy (Katz *et al* 1989). Many of the complications of pregnancy related to fibroids have been attributed to an increase in tumour size during the gestational period (Barter & Parks 1958). Some researchers have postulated that the increase in size of these tumours may be apparent rather than real and is possibly the result of easier palpation and identification in an upwardly expanding uterus (Randall & Odell 1943).

Tumours of less than 3cm in diameter do not appear to be clinically significant, while those of greater size are associated with an increase in the frequency of spontaneous abortion (Stevenson 1964), premature labour and pelvic pain (Rice *et al* 1989). The presence of pelvic pain during pregnancy has also been linked to 'red degeneration' of the fibroids (Randall & Odell 1943). The location of a leiomyoma, more especially its anatomical relationship to the placental site, appears to be even more significant than tumour size in the prediction of pregnancy outcome (Muram *et al* 1980).

Submucous fibroids significantly increase the risk of placental abruption with up to a 50% foetal mortality occurring when there is direct contact between the fibroid and the placenta (Rice *et al* 1989). The probability of post-partum haemorrhage and puerperal sepsis are also increased in the presence of submucous fibroids (Abitol 1957). Large fibroid tumours that distort the uterine cavity may result in uterine growth retardation, malpresentations, labour dystocia and retained placentae (Katz *et al* 1989, Rice *et al* 1989).

2) MANAGEMENT

The clinical management of leiomyomata is by no means standard and varies according to the severity of the symptoms, as well to the age and wishes of the patient. Asymptomatic tumours are usually discovered incidentally and as such require no treatment other than monitoring. The current forms of therapy available include both surgical and medical options.

2.1) *Surgical Management*

2.1.1) *Hysterectomy and Myomectomy*

Both hysterectomy and myomectomy form classical surgical methods for the management of leiomyomata. The indications for surgery usually include:

* Tumours with excessive growth rates
* Menorrhagia in the presence of submucous fibroids
* Pedunculated tumours
* Urinary tract pressure symptoms
* Other associated pelvic pathology
* Location of tumour predisposes to habitual abortion

Myomectomy is usually the operation of choice for those women under the age of 40 years who wish to maintain reproductive function (Brown *et al* 1967, Israel & Mutch 1958, Kimbrough 1958). Although myomectomy is often carried out in the hope of improving fertility (Lock 1969, Smith & Uhlir 1990), it is a highly complicated technique that may result in reduced rather than improved fertility (Loeffler & Noble 1970). The two major technical problems associated with the technique are the minimisation of blood loss and the prevention of adhesions (Te Linde 1958).

As opposed to myomectomy, hysterectomy is more commonly performed in women who have completed their families and are over the age of 40 years. This form of surgery is also preferred for the larger leiomyomata (Edwards & Beebe 1958), especially those over 14 weeks in size. The advantages of hysterectomy include the preclusion of recurrence (Malone 1969) and the cessation of symptoms. Unlike myomectomy, hysterectomy is both simpler to perform and blood loss more easily minimised (Counseller & Welch 1958).

2.1.2) *Resectoscopes and Lasers*

The ever increasing sophistication and availability of advanced instrumentation means that less invasive forms of surgical therapy are becoming a viable alternative to the more traditional methods (Lalonde 1994). Small submucous leiomyomata can be safely and effectively removed by hysteroscopic resection. The major complication associated with this form of therapy is fluid overload as a result of the large quantities of fluid that are pumped into the uterus to allow for good visualisation.

Alternatively laser myomectomy may be performed with direct vaporisation of small leiomyomata. Good haemostasis and fewer adhesions are achieved using this technique. Currently there are three types of lasers in use. These include Co_2, Argon and ND-YAG (Loffer 1987, McLaughlin 1985, Starks 1988).

2.2) *Medical Therapy*

The derivation of suitable pharmacological therapies for the management of leiomyomata is still in its infancy when compared to the surgical alternatives. Even so the long term usage of gonadotropin releasing hormone (GnRH) analogues has certainly become recognised as a possible future alternative to surgery (Editorial 1986).

The long term administration of GnRH analogues suppresses the pituitary secretion of gonadotropin producing a hypo-oestrogenic pseudomenopausal state McLachlan *et al* 1986). In response to this induced state of pseudocastration leiomyomata undergo major reductions in size (Kessel *et al* 1988). Almost complete regression has been recorded in the smaller fibroids (Maheux *et al* 1987) and up to a 50% reduction has occurred in the larger tumours (Coddington *et al* 1986, Pasqualini *et al* 1990).

Even so some leiomyomata have not undergone volume reduction even in the face of castration levels of oestrogen. It has been surmised that these particular tumours may not be oestrogen dependant. The cessation of therapy is unfortunately often accompanied by the regrowth of the tumours and a recurrence of symptoms (Friedman *et al* 1987, Matta *et al* 1989, West *et al* 1987).

It is of concern that GnRH therapy is not without its dangers. Prolonged periods of hypo-oestrogenism leave patients susceptible to conditions such as osteoporosis. This can be prevented by hormone replacement therapy, where an initial period of hypo-oetrogenism is followed by equine oestrogen therapy. It appears as if these oestrogens, given subsequent to the initial high doses of GnRH, do not provide sufficient stimulus for the regrowth of the tumours (Friedman 1989).

GnRH therapy is also a useful pre-operative method for reducing the menorrhagia associated with leiomyomata, so that a normal haemoglobin level can be restored prior to surgery (Donnez et al 1989, Friedman *et al* 1988).

Finally, although GnRH therapy has a definite place in the management of leiomyomata, at present it delays rather than abandons the surgical alternative (Editorial 1986).

CHAPTER FIVE

STUDY OUTLINE AND SAMPLE COLLECTION

1) STUDY JUSTIFICATION

Scientific endeavour related to leiomyomata is certainly justified when one considers that these tumours are extremely common and although benign, not innocuous. The complications related to their presence form a large percentage of gynaecological morbidity with major financial implications. The effect of media campaigns and an increasing awareness, by medical personnel, of a woman's right to choose between available therapies is affecting both the form and outcome of management related to leiomyomata (Domenighetti *et al* 1988). Thus in order to meet the realistic expectations of women, rational forms of therapy must be designed and offered. To this end a thorough understanding of the fundamental character of the tumour is essential. To gain such an understanding this thesis aims to answer the following questions:

1) Is the non-neoplastic myometrium of leiomyomatous uteri normal or abnormal?

2) What differences in structure and oestrogen receptor content are there between normal myometrium and leiomyomata?

3) Do leiomyomata and myometrium respond in a like manner to changes in age, parity and endogenous oestrogen?

2) STUDY OUTLINE

In order to answer the questions posed the study has been divided into two broad categories. Firstly, a structural analysis of normal and abnormal tissue using light and electron microscope techniques and secondly, a 'functional' analysis encompassing immunocytochemistry, radioimmunoassay and serum analysis.

Details of these analyses and each of the specific study designs are presented in the relevant chapters.

3) SAMPLE COLLECTION

3.1) *Patient sample*

A total of 191 surgically resected uteri was collected, over a one year period, from women undergoing hysterectomy at the Groote Schuur Hospital, Cape Town, South Africa. The women, of all races, ranged in age from 13 years to 76 years of age. Gravidity and parity for the patients ranged from 0 to 16 (median 3) and 0 to 13 (median 3) respectively. Full clinical notes were obtained from the hospital for each patient.

Just prior to the induction of anaesthesia, 10mls of venous blood was collected from each patient, placed into a sterile plain glass tube and allowed to clot. The uterus, once surgically removed and accompanied by the clotted blood, was immediately sent, unfixed, in a plastic container, to the pathology laboratory where tissue sampling was performed expeditiously. All uteri that took more than 10 minutes from the time of surgical removal to reach the laboratory, as well as those that arrived 'cold' to the touch, were not included in the sample. Also excluded from the study sample were uteri that demonstrated any evidence of pathology other than leiomyomata e.g. carcinoma of the cervix. The final composition of the sample, classified according to age, the phase of the menstrual cycle and the presence of the tumour, is summarised in Table 2.

Of the 191 uteri collected, 58% were from women between the ages of 41 and 50. This reflects the increased risk for the development of troublesome fibroids during the climacteric. Only 3 uteri were obtained from patients below the age of 20. All 3 were removed from severely mentally retarded girls due to 'a failure to cope with menstruation', as well as for contraceptive purposes. The uteri of the ladies over the age of 70 years were removed due to unexplained per vaginum bleeding and suspicion of malignancy. No neoplasia, other than leiomyomata in one, was noted in either of the two uteri.

In general the majority of hysterectomies were performed for menorrhagia, often in the absence of clinical anaemia, while a small number were performed for pelvic inflammatory disease and/or contraceptive reasons. Over 75% of uteri

included in the sample were leiomyomatous. This high percentage is a reflection of the sampling technique where all uteri other than those that appeared 'normal' or demonstrated the presence of leiomyomata, were excluded.

Table 2: Composition of study sample.

	Phase of Menstrual Cycle					
Age (years)	Proliferative	Secretory	Menstrual	Inactive	Tot.	Leiomyomata
< 20	1	2	0	0	3	0
21 - 30	3	2	0	2	7	3
31 - 40	18	7	6	5	36	21
41 - 50	45	26	6	34	111	72
51 - 60	6	6	1	12	25	14
61 - 70	3	0	0	4	7	3
> 70	0	0	0	2	2	1
Total	76 (39,7%)	43 (22,5%)	13 (6,8%)	59 (30,8%)	191	144 (75,3%)

In the patients with normal menstrual cycles, surgical removal was most commonly performed during the proliferative and secretory phases with only 6,8% of samples being obtained from hysterectomies performed during menses. The reason for this is two fold. Firstly, surgeons prefer not to operate during the menses as the reproductive organs are usually engorged with blood and haemostasis and subsequent blood loss may prove problematic. Secondly, the issue of menstruation is culturally sensitive and many women prefer not to be in hospital or examined while they are menstruating. The endometrium of almost one third of the uteri was 'flat' and inactive. For over 55% of the 'inactive' group this represented the histological response to medical therapy aimed at reducing menorrhagia. For many of these patients, even though the endometrium appeared inactive, hysterectomy was performed on the grounds of continuing clinical symptoms in the face of failed medical therapy.

3.2) *Tissue sampling*

Upon their arrival in the pathology laboratory, from theatre, the uteri were sectioned in the sagital plane, so that a left and right half of the uterus were produced. Three parallel transmural blocks of tissue were dissected from both the

fundus and lower segment from one half of each uterus. Following careful resection of the endometrium and serosa, two of the blocks from each region were further subdivided into three equal portions (Figure 9). These blocks were labelled subendometrial, middle and subserosal respectively, prior to one set from each region being snap frozen in liquid nitrogen while the remaining set was placed in 2% glutaraldehyde. The snap frozen tissue was subsequently stored in foil bags in a -70^{o}C freezer until analysed by radioimmunoassay (see Chapter 8). The tissue in glutaraldehyde was further processed for electron microscopy (see Chapter 7). The remaining transmural blocks of fundal and lower segment tissue, with the endometrium and serosa intact, were used for routine histology (see Chapter 7) and immunocytochemistry (see Chapter 8), subsequent to fixation in 10% formalin and processing through a series of graded alcohols and xylol to wax.

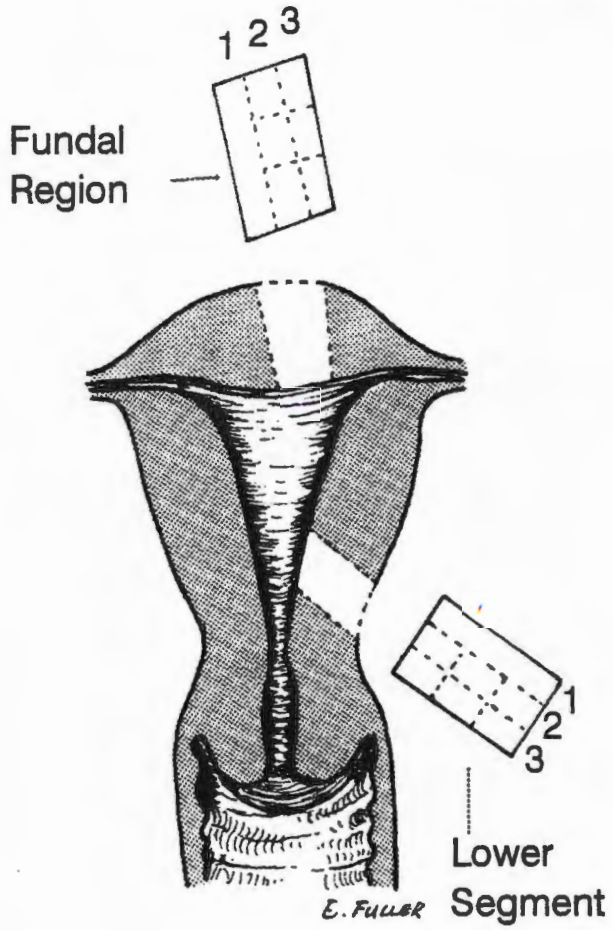

Figure 9: Schematic representation of a coronal section through the uterus to demonstrate tissue sampling. Where: (1) represents the tissue used for immunocytochemistry and (2) and (3) represent the samples used for radioimmunoassay and electron microscopy respectively.

The samples of clotted venous blood were centrifuged in order to separate the serum from the cellular components. The serum was then pipetted into cryotubes and stored at -70^{O}C until required for assay (see Chapter 6).

Where leiomyomata were present they were sampled and processed in exactly the same manner as the myometrium with the exception that they were not designated as 'fundal' or 'lower segment'. Fibroids over 3 cm in diameter were considered 'large' while those between 3 mm and 3 cm were deemed 'small'. Minute leiomyomata, i.e. less than 3 mm in diameter, were not sampled as these were not usually visible at gross dissection.

CHAPTER SIX

SERUM HORMONE ASSAYS

1) INTRODUCTION

That the serum levels of oestradiol and progesterone undergo cyclical variations according to the phase of the endometrial cycle is a well documented phenomenon (Adashi 1991, Yen 1991).

The proliferative phase of the cycle is characterised by low levels of progesterone and increasing levels of oestradiol, which peak just prior to ovulation. Oestradiol levels drop after ovulation but increase slightly during the mid-proliferative phase dropping again prior to menses. The serum levels of progesterone rise rapidly after ovulation and demonstrate two peaks. The levels then remain high until just prior to menses when they plummet (Adashi 1991).

Sex hormone binding globulin, a glycoprotein with a molecular weight of about 86 000, is synthesized by the liver and functions in the transport of the reproductive steroid hormones. About 37% of oestradiol is transported in the serum bound to this protein, whilst the majority of the remaining oestradiol is loosely bound to albumin. Only a small fraction of the total quantity of oestradiol is free or unbound (Yen 1991).

The serum levels for the reproductive steroid hormones are said to fall within normal limits in those patients with leiomyomata (Soules & McCarty 1982). However, as the primary purpose of this thesis is to compare normal and leiomyomatous uteri, it is essential that the parameters under which they are examined are as specific as possible. It is to this end that the following study is aimed.

2) SERUM HORMONE STUDY

2.1) *Study Aim*

To assess whether the circulating serum levels of oestradiol, progesterone and sex hormone binding globulin differ between patients with normal uteri and those with leiomyomatous uteri, in order to determine standards for further tissue sampling.

2.2) *Materials and methods*

2.2.1) *Patient Sample*

Samples of venous blood from a total of ninety patients were suitable for serum assay. The remaining samples were either insufficient in quantity for analysis or were not collected at the time of surgery. The samples drawn from the patients covered both the full range of ages and phases of the endometrial cycle.

2.2.2) *Methods*

Blood samples were collected and stored as detailed in chapter five. Oestradiol measurements were conducted by means of a specific radioimmunoassay (Appendix I:I) performed on extracts of the plasma samples. These utilised a rabbit anti-17-ß-oestradiol-6BSA serum from Biomaker and the radioactive tracer [2,4,6,7,16,17-^{3}H]-oestradiol (specific activity 140-170 Ci/mmol) from Amersham Laboratories. The cross reactivity of the antiserum relative to oestradiol was 100%, for oestrone 0,75%, 0,1% for oestriol and 0,2% for oestradiol-17-α. The percentage recovery of oestradiol from the plasma extracts was 82 $\pm$ 5% while the sample sensitivity for the assay was 51pmol/l.

Serum progesterone was measured using a Coat-A-Count^{R} Progesterone Kit (Appendix I.II). This is solid-phase radioimmunoassay where ^{125}I-labelled progesterone competes with patient progesterone for antibody sites which are immobilised to the wall of a polypropylene tube. Specificity for progesterone was 100%, for corticosterone 0,4% and 11-Deoxycortisol 2,4%. Sensitivity was calculated to be 0,0954nmol/l.

Serum sex hormone binding globulin (SHBG) was estimated using an immunoradiometric assay kit supplied by Orion Diagnostica. The procedure is based on the principles of a non-competitive liquid phase radioimmunoassay (Appendix I:III). The radioactive monoclonal antibody (mouse) was labelled with ^{125}I with a maximum activity of 3 μCi. Recovery of SHBG was 91 $\pm$ 6% and sensitivity was equal to 0,5nmol/l. The test was 100% specific and did not cross-react with any other antibodies.

2.3) *Results*

Serum oestradiol values are presented in Table 3. In patients with normal uteri the serum values all lie within the normal expected ranges for the relevant phases of the menstrual cycle, where oestradiol values are at their highest during the proliferative phase, with a marked decrease during secretory phase. As is to be expected the older patients demonstrate low circulating levels of oestradiol and inactive endometria. The serum oestradiol values for patients with leiomyomatous uteri mirror those of the normal patients (Figure 10). The Mann-Whitney U non-parametric test shows no significant difference, between the serum values of the normal and leiomyomatous groups, for any of the endometrial phases.

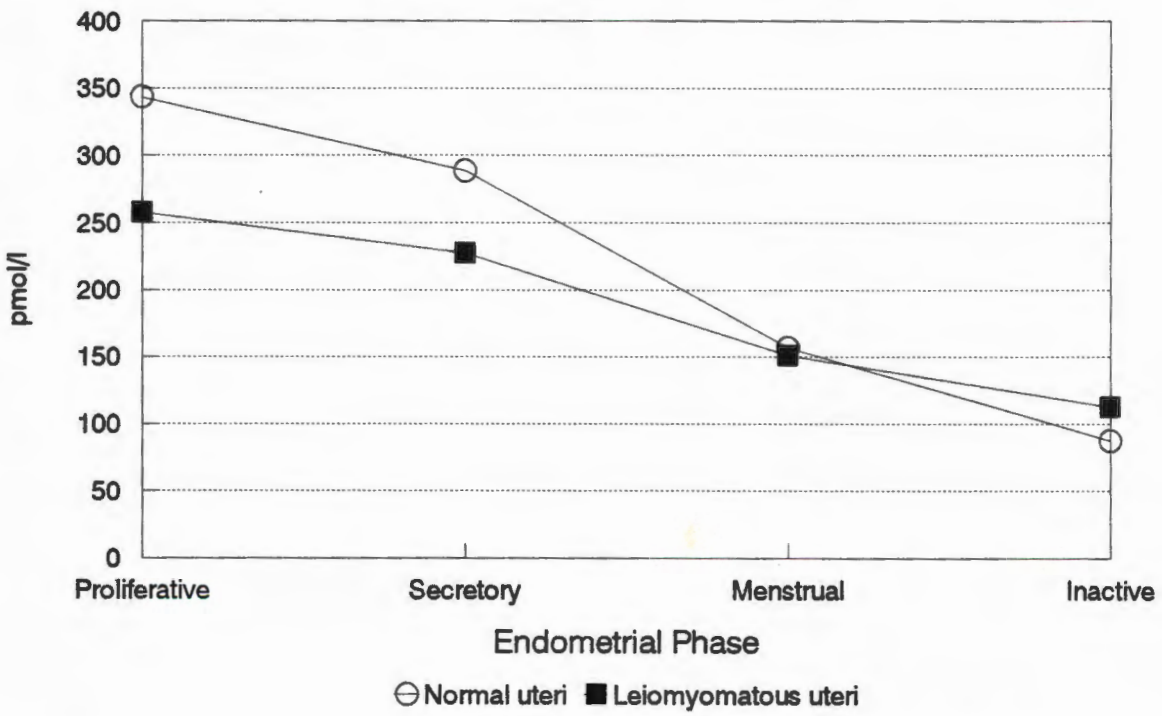

Figure 10: Graphical representation of serum oestradiol levels during the menstrual cycle.

The serum values obtained for progesterone are presented in Table 4. As in the case of oestradiol, progesterone values for the normal group fall within the normal expected range, where the hormone is markedly increased during the secretory phase. A similar pattern is recorded for those patients with leiomyomatous uteri (Figure 11) where progesterone levels increase after ovulation. The serum progesterone levels of leiomyomatous patients, with inactive endometria, are significantly greater than those of the normal patients. This is due to all, bar one, of the leiomyomatous group having received progestagen therapy prior to surgery. None of this group were treated with medroxy-progesterone but with either a combined or sequential preparation.

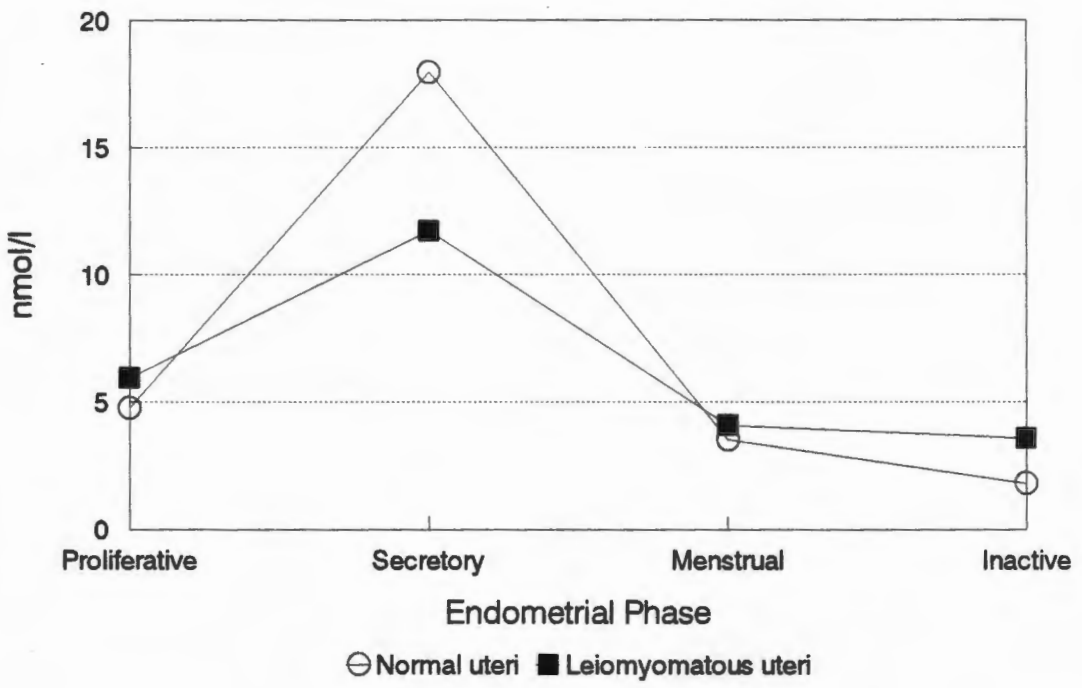

Figure 11: Graphical representation of serum progesterone levels during the menstrual cycle.

All of the values obtained for serum SHBG, whether from the normal or the leiomyomatous group, fall within the normal expected range of 8-109nmol/l (Table 5). No statistically significant difference exists between the two groups for any of the phases of the menstrual cycle (Figure 12). The mean SHBG values do not vary significantly with the phase of the cycle but do tend to decrease after the climacteric.

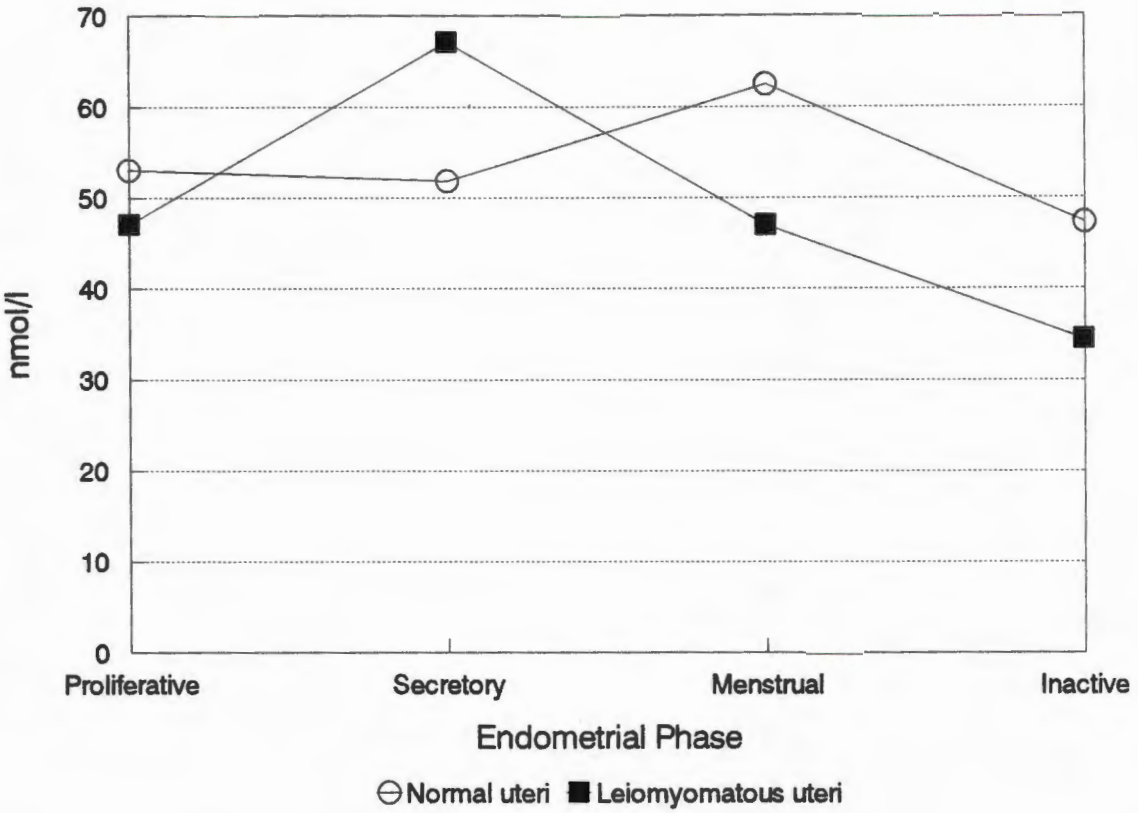

Figure 12: Graphical representation of serum SHBG levels during the menstrual cycle.

2.4) *Discussion*

Both the present study and other researchers have shown that the normal cyclical pattern of the reproductive steroid hormones applies to both normal and leiomyomatous patients (Runnebaum *et al* 1978, Soules & McCarty 1982, Spellacy *et al* 1972).

The fact that the values for the SHBG are normal for both groups of tissue suggests that the transport mechanism for oestradiol is unaffected in leiomyomatous patients. This combined with the normal serum values for oestradiol and progesterone, in the proliferative, secretory and menstrual phases, means that the tumours are not initiated by abnormal levels in the circulating reproductive steroids.

The significant difference that exists between the serum levels of progesterone, in normal and leiomyomatous patients with inactive endometria, is attributable to pre-surgical therapy aimed at reducing leiomyoma-associated

menorrhagia and restoring normal haemoglobin levels. The mean serum value for the leiomyomatous group is twice that of the normal group and reflects the type of preparation used. Medroxy-progesterone therapy results in massive increases in the serum progesterone levels while combined and sequential preparations produce the elevations recorded in this study.

2.5) *Conclusion*

The circulating serum levels of oestradiol, progesterone and SHBG are similar for both normal and leiomyomatous patients with normal endometria. Exogenous progestagen therapy significantly alters the circulating levels of progesterone and produces flat inactive endometria. As the underlying purpose of this thesis is compare normal and leiomyomatous uteri, at a baseline level, it is essential to eliminate as far as possible any variable parameters that may affect that comparison. It is for this reason that all patients with a history of exogenous hormone therapy are to be excluded from the remainder of the studies.

Table 3: Serum Oestradiol Values (pmol/l)

	NORMAL UTERI				LEIOMYOMATOUS UTERI			
Endometrial Phase	Proliferative n=13	Secretory n=12	Menstrual n=4	Inactive n=10	Proliferative n=22	Secretory n=12	Menstrual n=5	Inactive n=11
	70	351	138	88	294	207	102	77
	758	384	168	69	113	124	114	70
	234	409	188	87	186	365	165	94
	124	193	134	92	162	274	100	195
	199	406		66	140	309	274	121
	546	129		49	92	361		103
	184	204		156	153	102		75
	257	232		88	361	315		75
	587	220		105	416	257		169
	334	97		72	205	188		87
	770	230			227	146		171
	210	609			108	80		
	190				846			
					274			
					160			
					206			
					246			
					507			
					251			
					197			
					260			
					264			
Mean	343.31	288.67	157.00	87.20	257.64	227.33	151.00	112.45
Std Dev	238.79	145.77	25.64	28.96	165.52	100.41	73.65	45.23
Max	770	609	188	156	846	365	274	195
Min	70	97	134	49	92	80	100	70

The following p values were obtained, using the Mann-Whitney U non-parametric test, to determine whether there was a significant difference between the normal and leiomyomatous uteri during each phase of the menstrual cycle. Proliferative p = 0.44 (ns), Secretory p = 0.31 (ns), Menstrual p = 0.39 (ns) and Inactive p = 0.18 (ns).
s - significant difference, ns - no significant difference

Table 4: Serum Progesterone Values (nmol/l)

	NORMAL UTERI				LEIOMYOMATOUS UTERI			
Endometrial Phase	Proliferative n=13	Secretory n=12	Menstrual n=3	Inactive n=10	Proliferative n=24	Secretory n=12	Menstrual n=2	Inactive n=11
	4.7	27.2	4.4	2.4	32.8	5.4	4.9	3.3
	7.3	5.2	1.0	1.0	4.3	8.5	3.3	3.6
	6.3	6.0	5.2	1.0	5.0	3.0		4.8
	3.5	19.4		1.0	3.5	7.0		3.5
	6.8	13.9		1.7	5.7	11.1		5.7
	3.7	7.2		4.2	3.8	5.3		2.9
	6.0	15.7		1.9	17.2	5.4		2.1
	3.9	25.4		2.2	4.8	31.7		2.8
	4.1	13.4		1.0	2.1	29.7		5.5
	4.9	16.1		1.7	4.6	9.2		1.0
	4.2	32.4			2.7	6.7		4.3
	5.4	33.6			3.1	17.6		
	1.2				3.6			
					14.9			
					3.7			
					6.1			
					1.8			
					5.5			
					2.2			
					2.4			
					3.2			
					1.2			
					3.9			
					4.6			
Mean	4.77	17.96	3.53	1.81	5.95	11.72	4.10	3.59
Std Dev	1.63	9.83	2.23	0.99	6.82	9.62	1.13	1.42
Max	7.3	33.6	5.2	4.2	32.8	31.7	4.9	5.7
Min	1.2	5.2	1.0	1.0	1.2	3.0	3.3	1.0

The following p values were obtained, using the Mann-Whitney U non-parametric test, to determine whether there was a significant difference between the normal and leiomyomatous uteri, during each phase of the menstrual cycle. Proliferative p = 0.26 (ns), Secretory p = 0.10 (ns), Menstrual p = 1.00 (ns) and Inactive p = 0.005 (s).
s - significant difference, ns - no significant difference.

Table 5: Serum Sex Hormone Binding Globulin Values (nmol/l)

	NORMAL UTERI				LEIOMYOMATOUS UTERI			
Endometrial Phase	Proliferative n=14	Secretory n=12	Menstrual n=3	Inactive n=13	Proliferative n=18	Secretory n=9	Menstrual n=4	Inactive n=9
	47.5	91.2	25.5	52.2	25.8	45.9	41.8	27.6
	25.8	69.3	57.3	50.9	13.0	17.7	76.7	72.3
	44.0	55.2	104.6	24.5	97.8	71.9	40.0	31.9
	55.7	16.0		88.5	58.1	93.3	29.4	27.4
	79.6	20.8		28.2	39.4	45.7		58.8
	73.4	36.3		42.3	80.4	27.3		4.6
	107.5	70.0		26.4	31.1	106.1		14.5
	18.9	52.6		71.9	32.2	47.5		40.2
	72.1	46.0		22.0	74.3	148.5		32.4
	28.5	69.8		36.0	7.3			
	21.2	53.0		77.1	43.6			
	33.5	42.1		47.3	30.3			
	54.2			47.4	67.4			
	80.8				44.7			
					44.6			
					74.2			
					50.3			
					41.3			
Mean	53.05	51.86	62.47	47.28	47.54	67.10	46.98	34.41
Std Dev	26.67	21.61	39.80	21.10	23.97	42.09	20.56	20.78
Max	107.50	91.20	104.60	88.50	97.80	148.50	76.70	72.30
Min	18.90	16.00	25.50	22.00	7.30	17.70	29.40	4.60

The following p values were obtained, using the Mann-Whitney U non-parametric test, to determine whether there was a significant difference between the normal and leiomyomatous uteri, during each phase of the menstrual cycle. Proliferative p = 0.65 (ns), Secretory p = 0.55 (ns), Menstrual p = 0.86 (ns) and Inactive p = 0.23 (ns).
s - significant difference, ns - no significant difference.

CHAPTER SEVEN

MORPHOLOGICAL STUDIES

1) INTRODUCTION AND STUDY DESIGN

Both the gross anatomical structure and histological features of normal myometrium and leiomyomata have been extensively described in the literature (Chapters One and Three). In comparison there is a dearth of available basic studies of these tissues at the electron microscope level. This is probably due to the fact that other than an abnormal architectural arrangement and degenerative changes, leiomyomata and normal myometrium are histologically very similar. Also, many of the ultrastructural studies of myometrium and leiomyomata have been published in scientific journals in national languages and as such are not easily available, in translated form, in South Africa. In the English language many of the basic ultrastructural descriptions of these tissues have been provided by Ferenczy and co-workers (Ferenczy 1979, Ferenczy *et al* 1971, Ferenczy & Richart 1974) and are discussed in Chapters One and Three.

The very nature of biological tissue means that states of normality are variable and will change according to alterations in the biological system and/or organism of which the tissue forms part and interacts with. The following ultrastructural studies are aimed at firstly, defining 'normality' in both unaffected and leiomyomatous myometrium. Secondly, tumour tissue structure is compared to the base-lines for normality, as defined in the first study. Finally, the anatomical response, to increasing age and parity, of both normal and tumorous tissue is examined.

The results of these morphological studies are presented in the form of micrographs with an accompanying description of the relevant observations. In order to convey a better understanding of these observations, to the reader, a discussion, citing the literature, has been included concomitantly with the presentation of the results.

2) MORPHOLOGICAL STUDY ONE

2.1) *Study Aim*

This study is aimed at establishing whether the myometrium of leiomyomatous uteri is normal or abnormal at the ultrastructural level. The results of the study should provide an indication of whether or not it is possible that leiomyomata arise as a result of a primary structural abnormality in the myometrium in which they occur.

2.2) *Materials and Methods*

2.2.1) *Patient Sample*

The uteri selected for the study were divided into two groups. Firstly, those that appeared normal on gross anatomical examination and secondly, those that were clearly leiomyomatous. Both groups were further limited to those uteri excised from women between the ages of 31 - 50 years in order to minimize any possible effects that age (either young or old) may have on the ultrastructure of myometrium. Furthermore the study sample included only those uteri of patients with known menstrual cycles and whom had been positively identified as within the proliferative, secretory or menstrual phases as at the time of surgery. The rationale for such a selection was to eliminate, as far as possible, any structural response to the onset of the climacteric and the accompanying changes in serum reproductive hormone levels. Any uteri demonstrating histological evidence of exogenous hormone therapy were excluded. A final sample of seventy seven uteri were used for the purposes of this study.

2.2.2) *Methods*

The fundal and lower segment tissue excised from the selected uteri (Figure 9) and embedded in paraffin wax, was subsequently sectioned using a manual sledge microtome. The $\pm$ 1 μm sections were then placed on glass slides, dewaxed and stained with haematoxylin and eosin (Appendix I:IV). The slides were examined by brightfield microscopy in order to confirm the histological normality of the tissue, the phase of the endometrial cycle (Gompel & Silverberg 1994) and the anatomical position of sampling (fundus or lower segment).

A total of 462 samples of myometrial tissue were fixed in a paraformaldehyde and glutaraldehyde fixative (Appendix II:I) prior to being processed to resin for transmission electron microscopy, according to the standard protocol presented in Appendix I:V. Ultra thin sections from each resin block were obtained using a Reichart Ultracut E ultramicrotome and stained with uranyl acetate and lead citrate (Appendix I:VI) prior to being viewed with a Hitachi H600 transmission electron microscope.

Ultrastructural examination of myocytes and the intervening extracellular matrix was carried out by 'blind' analysis. In other words, sections were viewed without knowing whether they were from normal or abnormal uteri.

2.3) *Results and Discussion*

2.3.1) *Phase of Endometrial Cycle*

A review of the literature indicates that oestrogen and progesterone can and do produce structural changes in the myometrium. Oestrogen when administered alone, at high or low dose, in either prepubertal or castrated rats, produces changes similar to those seen at oestrous (Ross & Klebanoff 1967). These alterations in structure may be seen within 24 hours of dosage (Friederici & DeCloux 1968) and include enlargement and dilatation of the endoplasmic reticulum as well as an increase in quantity of free ribosomes and interstitial collagen. The golgi complex and plasmalemmal vacuoles also become more prominent (Bo *et al* 1969, Azzopardi & Zayid 1967). All of these changes reflect an increase in the protein synthesizing capacity of the myocyte.

In contrast, when potent synthetic progesterones are administered to patients, the myometrium undergoes hypertrophy and the endometrium becomes predecidualised (Dito & Batsakis 1961). Within the rat, high dose progesterone produces an increase in the myofilament content of the myocyte. These alterations in structure mimic those changes that occur in association with the high levels of progesterone that are maintained during pregnancy. These morphological changes are aimed at the expulsion of the foetus from the uterus during parturition (Laguens & Lagrutta 1964).

In all of the above studies the doses of hormone administered were well above the normal physiological level. Also, many of the studies involved prepubertal or castrated animals and so eliminated the normal physiological interaction between oestrogen and progesterone. Thus while it is theoretically possible that oestrogen and progesterone could induce some structural changes in myometrium during the pre-ovulatory and post-ovulatory phases respectively, the serum levels of reproductive steroid hormones in normally cycling women are usually balanced against each other. Thus neither hormone should ever be in sufficiently high dose, in the non-pregnant female, for long enough to produce such a marked change.

This hypothesis is borne out by the results of the study where sections of myometrium, reflecting the three phases of the menstrual cycle, from both the normal and leiomyomatous uteri, are indistinguishable from one another, in the absence of endometrium, at both the light (Figures 13 and 14) and electron microscope level.

2.3.2) *Different Anatomical Areas*

The relative concentration of muscle fibres to intervening connective tissue appears to be greater in the fundal region than that seen in the lower uterine segment. This distribution is only noticeable at low magnifications and is similar for both groups of tissue (Figure 15). The inherent distribution of muscle fibres is related to the strong uterine contractions that are set up during the process of parturition in order to expel the foetus through the dilated, softened cervix.

In both normal and leiomyomatous uteri the subendometrial tissue is consistently more cellular than that of the subserosa (Figure 16)(Richards *et al* 1994). Whether such a distribution is the result of a hormonal influence or a structural preparation for an increase in uterine size during pregnancy, is unknown.

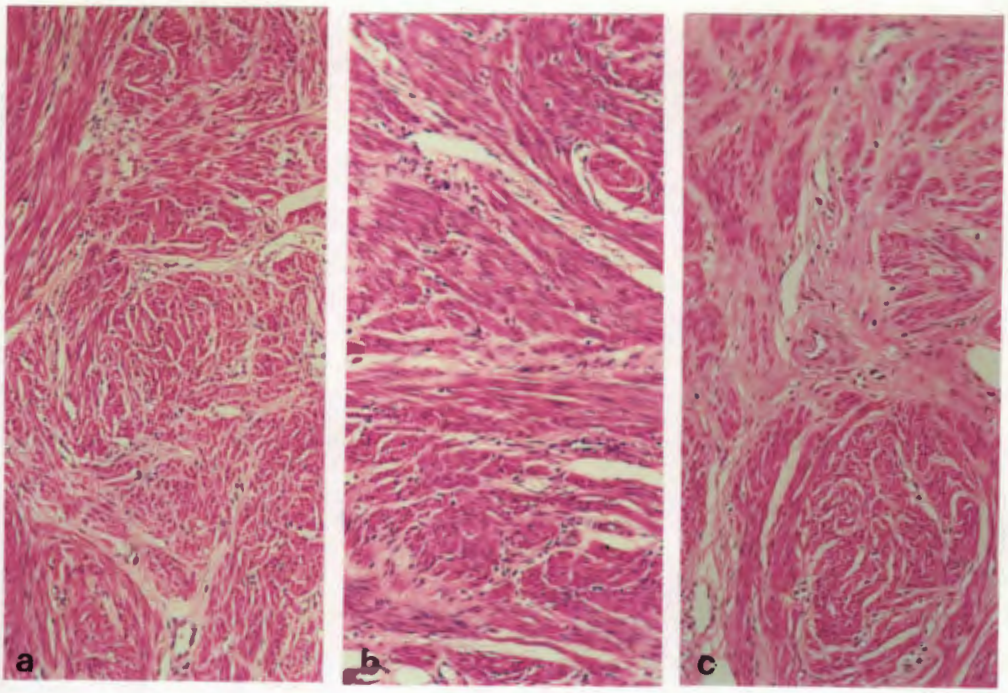

Figure 13: Light micrographs of H&E stained normal myometrium in (a) proliferative (b) secretory and (c) menstrual phase. Print magnification 110x.

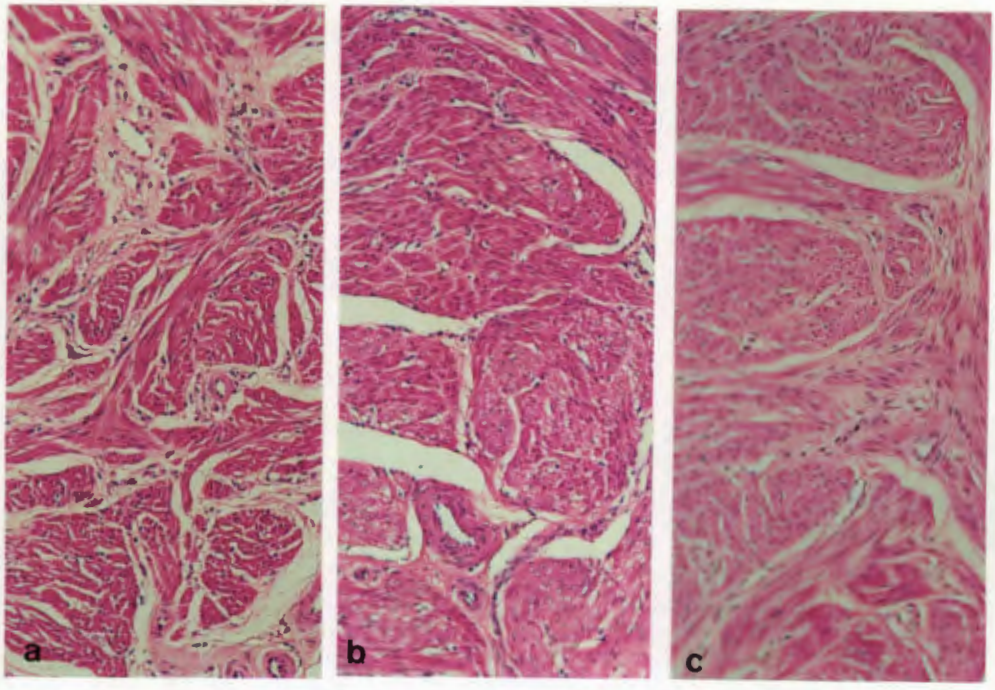

Figure 14: Light micrographs of H&E stained leiomyomatous myometrium in (a) proliferative, (b) secretory and (c) menstrual phase. Print magnification 110x.

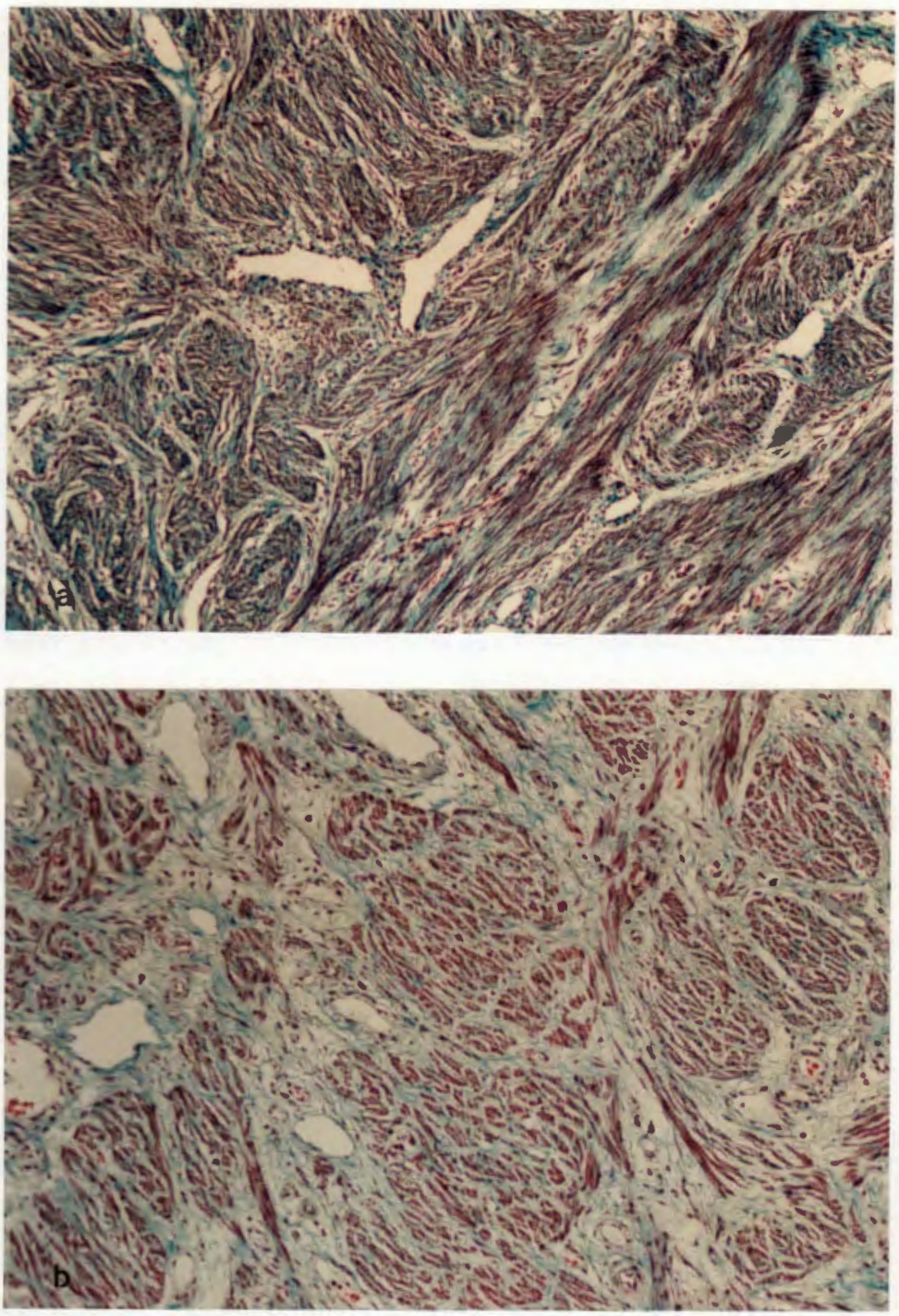

Figure 15: Masson's trichrome stained sections of normal myometrium from (a) the fundal region and (b) the lower segment showing the relative distributions of smooth muscle (red) to collagen (green). Print magnification 110x.

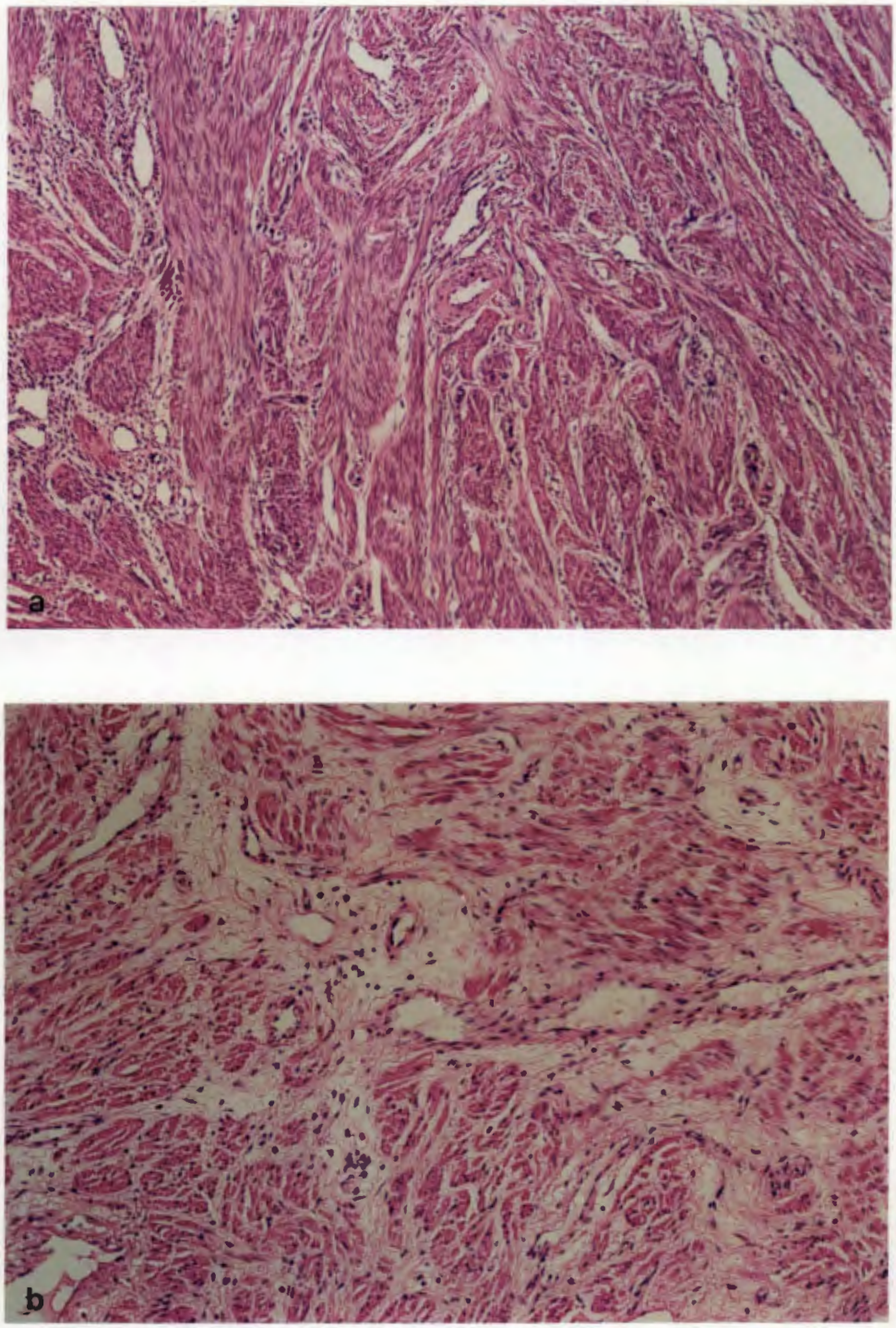

Figure 16: Light micrographs of H&E stained normal myometrium from the (a) subendometrial and (b) subserosal regions of the fundus. Note the higher degree of cellularity in the subendometrial zone. Print magnification x110.

2.3.3) *Specific Ultrastructure*

As no overt ultrastructural differences were noted between the normal and leiomyomatous myometria the descriptions which follow are applicable to both groups of tissue.

Nuclear Structure

At low magnifications, myocytes, cut in the x, y or z plane, are seen grouped together in fasciculi with varying amounts of intervening connective tissue (Figure 17). Smooth muscle nuclei (Figure 17) are round to ovoid in cross section and elongated with flattened tapered ends when cut longitudinally. The nuclei of the myocytes show varying degrees of indentation. There is usually only one nucleolus present but two are occasionally noted. Although the genetic material is dispersed, predominantly in the form of euchromatin, some perinuclear clumping does exist. The double nuclear membrane is distinct even though the thick inner membrane is often partially obscured by the nuclear material (Figure 20). Nuclear size is highly variable and dependant on the plane of section.

Organelles

The sarcoplasm of the myocyte contains the usual complement of cellular organelles. They are sparse and perinuclear in position (Figure 18). The mitochondria have a typical ultrastructural appearance with a double outer membrane and well defined internal cristae. Even though the fixation of the tissue is generally good some 'empty' mitochondria are seen (Figure 18).

Due to the plane of section, the golgi apparatus is not visible in every myocyte. When present, it has a bilaminated membrane which is centrally bowed with peripheral cysternal dilatations (Figure 18). Multiple small vesicles are found in association with the golgi apparatus and relate to the ability of the myocyte to produce and secrete the proteins associated with the extracellular collagen matrix (Bo *et al* 1969). Centrioles were seen in only a few of the total number of sections viewed and in each case they were located in close proximity to the golgi apparatus. The scarcity of these structures may be attributed to both the plane of section and the lack of mitotic figures. Although Goranova and Chaldakov (1990) have reported

on the presence of ciliated myocytes and trans-golgi-cilia complexes in the rat uterus, no such structures were noted in human myometrium.

Both smooth and rough endoplasmic reticulum are present, lying close to either the nuclear poles or the sarcolemma and are orientated parallel to the length of the myocyte. The quantity of endoplasmic reticulum varies from cell to cell and forms an anatomical indication of the degree of cellular activity related to protein production. Both ribosomes and glycogen granules are present but are difficult to distinguish from one another (Figure 20a).

Lipid-rich Residual Bodies and Lipofuscin

Paranuclear lipid-rich residual bodies are a relatively regular feature in both normal and leiomyomatous myometrium and may be located at either one of the nuclear poles (Figure 19). Structurally they are circular in section and range from 0,5 to 4μm in diameter. They are bounded by a single outer limiting membrane with a submembrane space of uniform width. The outer membrane encloses a single homogeneous lipidic inclusion of uniform electron density. Similar inclusions were documented by Pourcho and co-workers (1979) in oestrogen stimulated mice but these were not membrane bound and were also found in the interstitium (Figure 18b). Eyden *et al* (1991) suggest that these inclusions may appear as the result of degradation of the excess intracellular membranous structures that are produced in response to the normal oestrogenic stimulation of the myometrium, during the proliferative phase of the menstrual cycle. No clearly visible increase in the membranous structures of proliferative phase myometrium was noted in this study. The light microscopic identification of cathepsin D, a lysosomal enzyme whose production and function is regulated by oestrogen, within the lipid inclusions helps confirm their degradative nature (Yamazaki *et al* 1993). Electroncytochemical localisation of cathepsin D is still required in order to confirm that the presence of the enzyme is associated with the lipid structures identified at the electron microscope level.

A small number of lipofuscin bodies are also seen in the sample viewed (Figure 19). These are structurally distinct from the lipid-rich residual bodies in that they tend to be larger, have a high proportion of granular matrix and are not always homogeneous. Although Eyden *et al* (1991) describes some lipofuscin-like structures as lipid-rich residual bodies he does acknowledge that the two entities are intimately related. It is perfectly probable from the evidence supplied in the

literature (Eyden *et al* 1991, Pourcho *et al* 1979, Yamazaki *et al* 1993), as well as from the results obtained in morphological study three, that what is described as a lipid-rich residual body is merely an immature form of lipofuscin.

Intracellular Filaments

Myofilaments fill most of the cytoplasm and are orientated parallel to the length of the myocyte (Figure 19). The individual filaments lie close to each other and along their length are numerous dense bodies analogous to the z-line structures of skeletal muscle cells (Mark 1956). The myofilaments have been positively identified as actin and myosin and are well described in the literature (Lowy & Small 1970).

Small amounts of intermediate filaments are seen between the perinuclear organelles (Figure 18)(Uehara *et al* 1971) and occasionally as small aggregates within the sarcoplasm. The intermediate filaments are thicker than the actin filaments, thinner than myosin (Leoni *et al* 1990) and often have a random arrangement. Within the cells of the myometrium the intermediate filaments that have been identified by immunocytochemistry include desmin, vimentin (Evans et al 1983), α-smooth muscle actin (Eyden *et al* 1992) as well as a group of cytokeratins in the 39-50 kd range (Brown *et al* 1987). The distribution of the intermediate filaments in the myocytes of normal and leiomyomatous myometrium allows for the interaction of these filaments with the other cytoskeletal elements and thus enables the appropriate functioning of the cytoskeleton.

Plasmalemmal Vesicles

Along the length of the plasmalemma, of myocytes from normal uteri, are numerous small plasmalemmal vesicles (Figure 20a). These vesicles, which are merely infoldings of the plasma membrane, interdigitate with the plasmalemmal densities and are related to the active transport of those elements important in the generation of muscle action potentials (Kao 1977). In the sections of leiomyomatous myometrium it appears, on qualitative assessment, as if the plasmalemmal densities occupy more plasmalemmal space (Figure 20b), than that occupied within their normal counterparts. Should this be the case then the relative number of plasmalemmal vesicles would be decreased in these uteri. Consequently the normal contracting mechanism in affected uteri could be compromised and may offer an explanation for certain clinical abnormalities that have been associated with the

presence of leiomyomata. These include infertility due to improper transport of sperm during intercourse as well as dystocia.

Plasmalemma

A typical trilaminar plasmalemma bounds each myocyte. The sarcolemmal gaps described by Mark (1956) are not noted and may well have been artifactual. A well defined, though not complete, basement membrane surrounds each myocyte.

Cell Junctions

Intracellular connections in the form of gap junctions occur with increasing frequency in the myometrium during pregnancy and at parturition (Garfield & Hayashi 1980). These connections are identified as areas where the cell membranes lie closely opposed to each other separated by a 2nm gap bridged by connecting structures. There are often opposing plasmalemmal dense bodies at these points (Figure 21). The function of gap junctions in the myometrium is to lower current impedance and thus encourage synchronised contraction (Garfield & Hayashi 1980). The finding of the occasional gap junction in both normal and leiomyomatous myometria is not unexpected when one considers that the uterus does contract in a rhythmical fashion during menstruation as well as during sexual intercourse. Garfield and Hayashi (1980) reported on finding such junctions during menstruation and suggested that they may be the response to a pathologic stimulus. Their presence in women with normal menstrual cycles and no overt pathological processes tends to rule this possibility out.

Extracellular Matrix

The extracellular matrix consists of predominantly collagen (Figure 17) with only small amounts of elastin. Numerous small calibre vessels are apparent (Figure 17). These are mostly venous rather than lymphatic and are identified by the presence of erythrocytes within their lumina. Mast cells are seen frequently in close proximity to the capillaries (Figure 17)(Fox & Abell 1965). The shape of the mast cells varies from round to elongated depending on the plane of section and the density of the surrounding connective tissue. These cells, whether in normal or leiomyomatous myometrium, display a multitude of different electron microscopic appearances. Common to all are numerous intracellular electron dense granular substructures and a large central nucleus. Qualitative assessment of the tissue does

not reveal any overt differences in the quantity or structure of the mast cells in relation to the phase of the menstrual cycle. However, variation of mast cell structure, within the endometrium, in relation to cycle phase has been documented (Drudy *et al* 1991). To date the function of the mast cells in relation to myometrium is undefined (Maluf & Gersell 1994).

2.4) *Conclusion for Morphological Study One*

A single but significant structural difference is noted between the normal and leiomyomatous myometrium. This difference takes the form of increased plasmalemmal densities in the myocytes of fibroid affected uteri. Although leiomyomata may not arise as a direct consequence of this structural difference its very existence suggests that the leiomyomatous myometrium is not inherently normal.

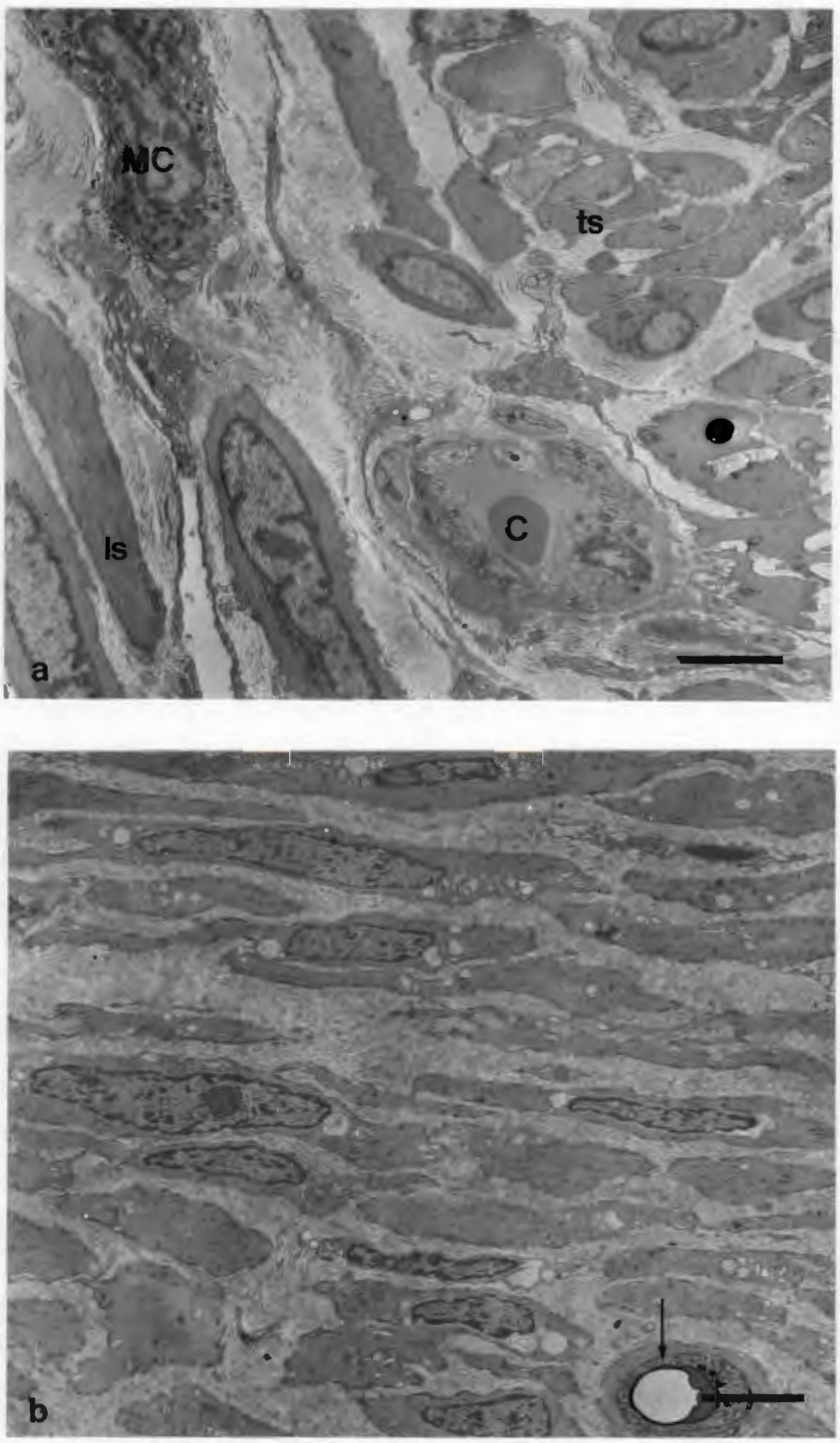

Figure 17: Low magnification electron micrographs of (a) normal and (b) leiomyomatous myometrium. Myocytes in longitudinal (ls) and transverse (ts) section are visible. A capillary (C) and mast cell (MC) are seen in close proximity to each other. Within the leiomyomatous section is a single lipid-rich residual body (arrow). Scale bar = 5μm.

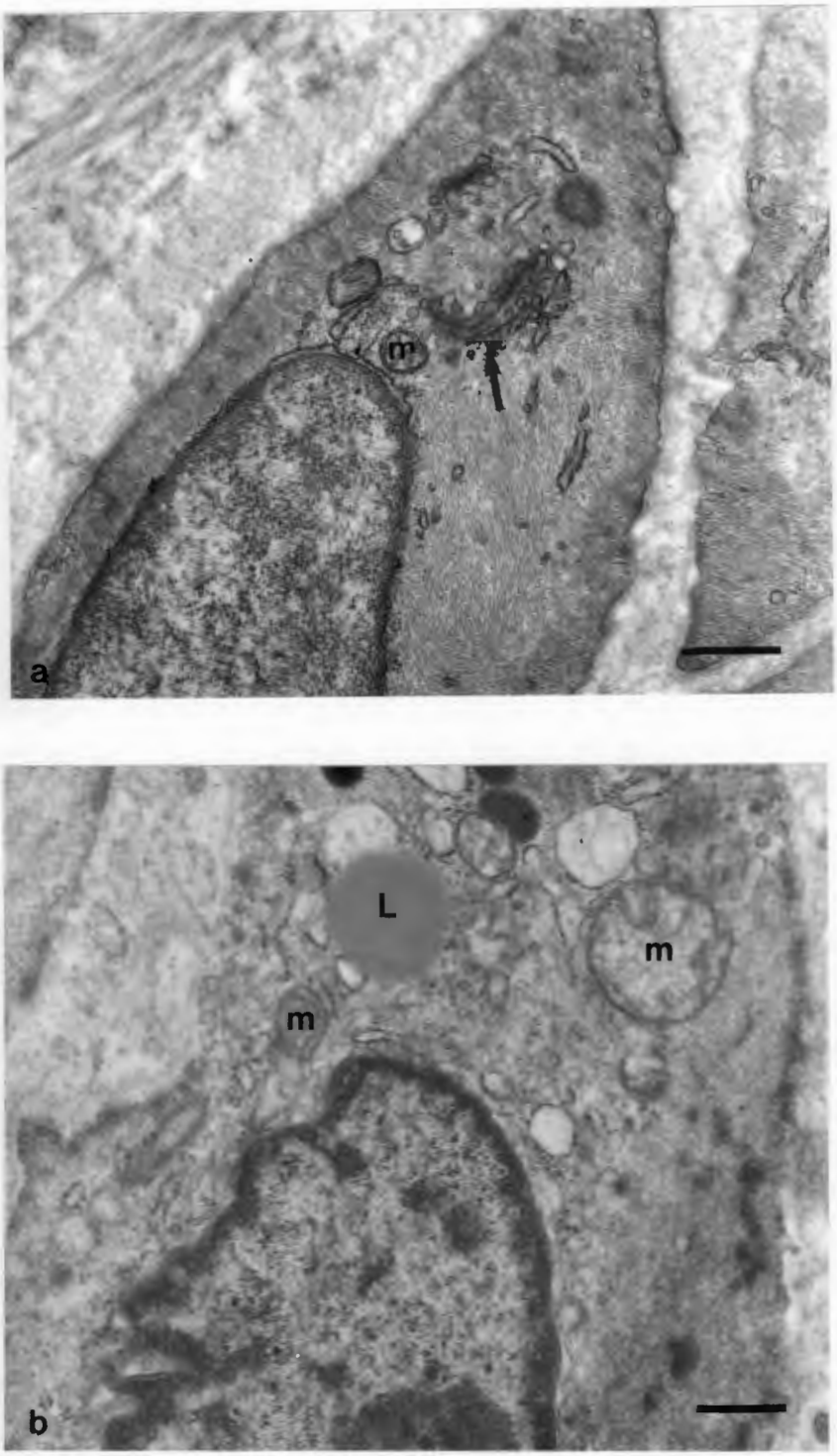

Figure 18: Electron micrographs demonstrating the perinuclear distribution of organelles in (a) normal and (b) leiomyomatous myometrium. Mitochondria (m), both well and poorly preserved, are present along with a golgi body (arrow) and a non-membrane bound lipid droplet (L). Wavy intermediate filaments are interspesed between the organelles. Scale bar = 0,5μm.

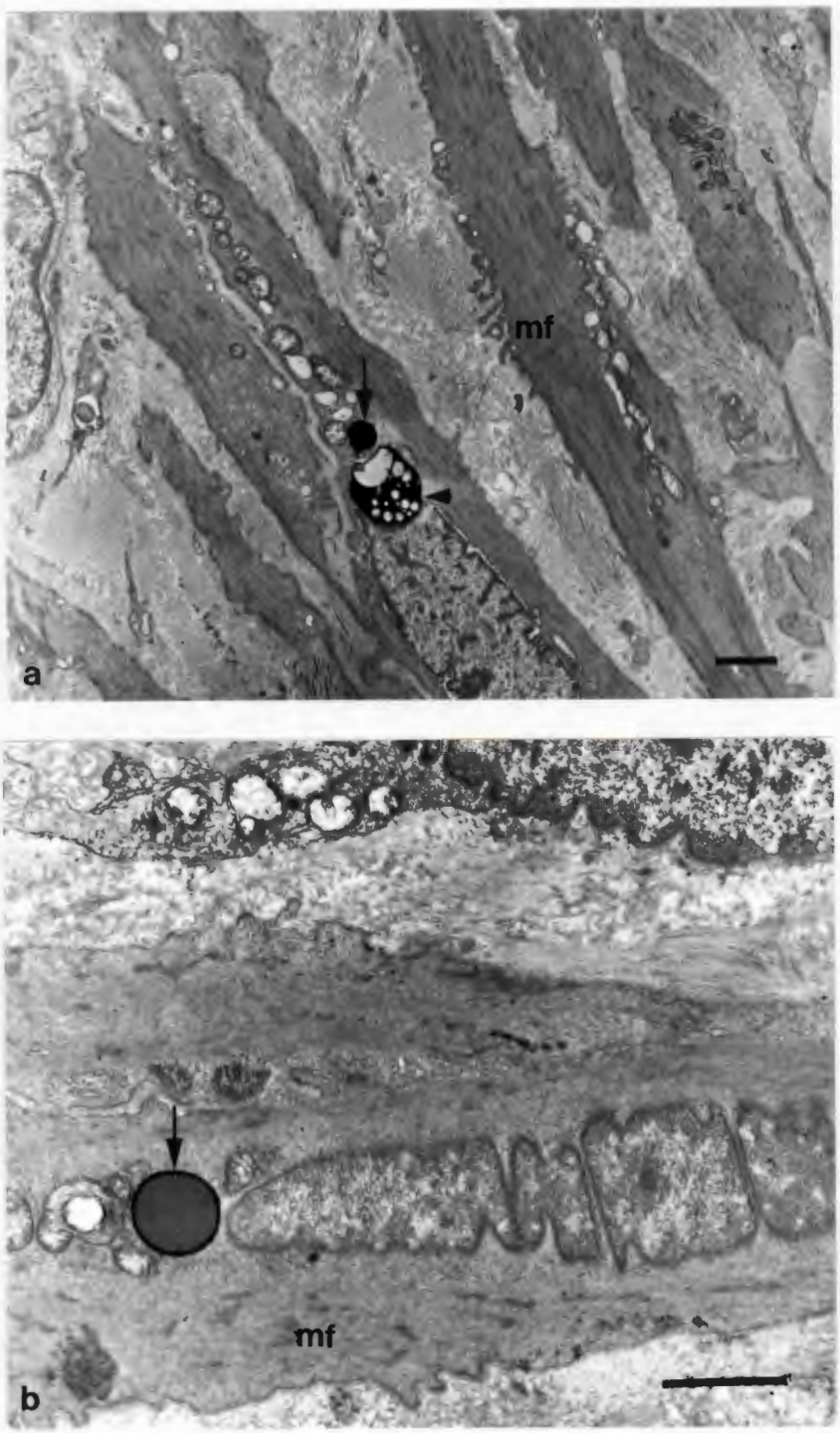

Figure 19: Homogeneous electron dense lipid residual bodies (arrows) as well as distinct myofilaments (mf) and dense bodies are noted in both (a) normal and (b) leiomyomatous myometria. A lipofuscin body (arrowhead) is located in the perinuclear region of the normal myocyte. Scale bar = 2μm.

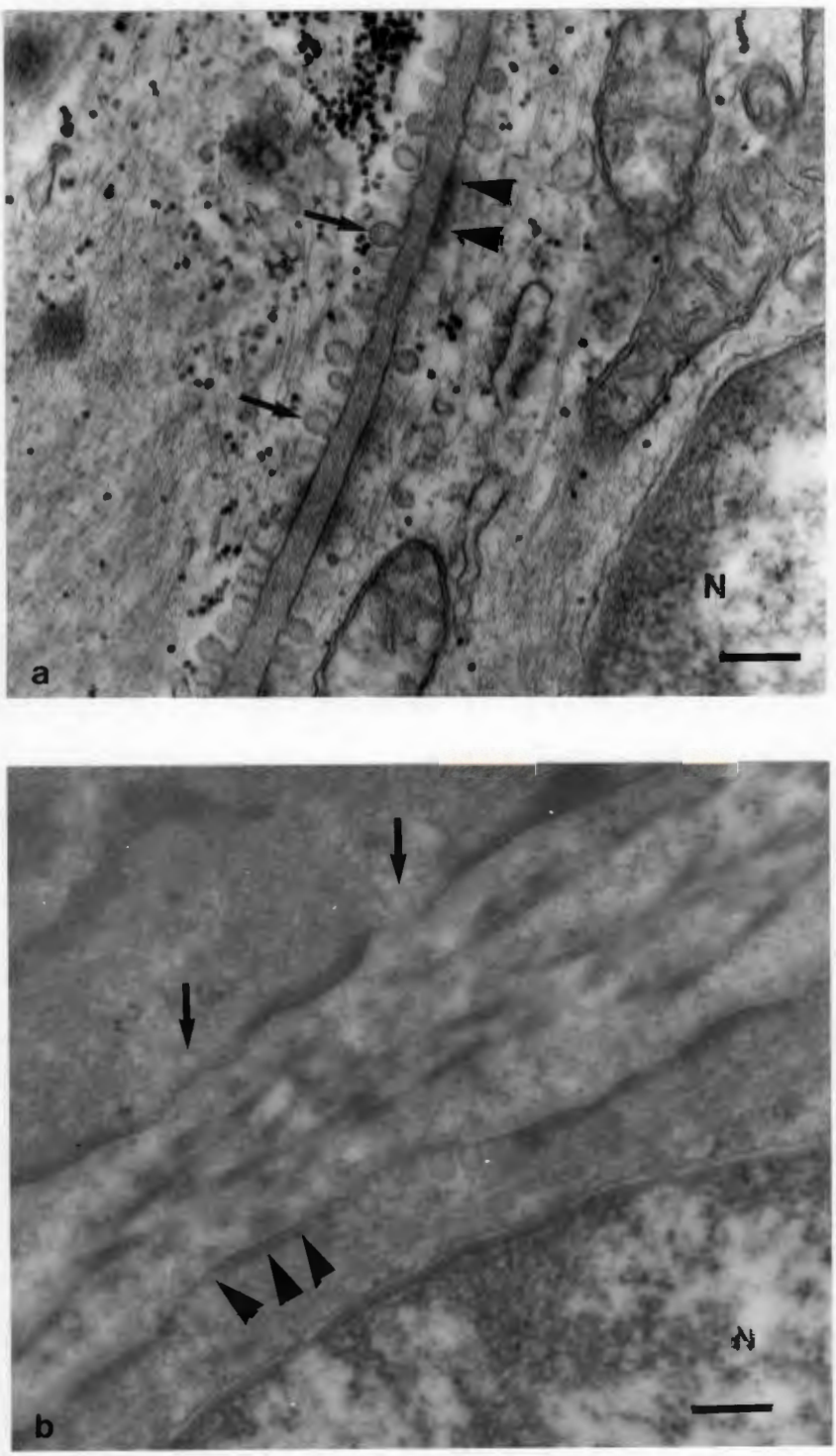

Figure 20: (a) Normal myometrium with distinct glycogen granules and ribosomes. Along the length of the sarcolemma numerous vesicles (arrows) are interspersed with plasmalemmal dense bodies (arrowheads). The densities appear to be longer in (b) the leiomyomatous myometrium with fewer intervening vesicles. N - Nucleus. Scale bar = 0,25μm.

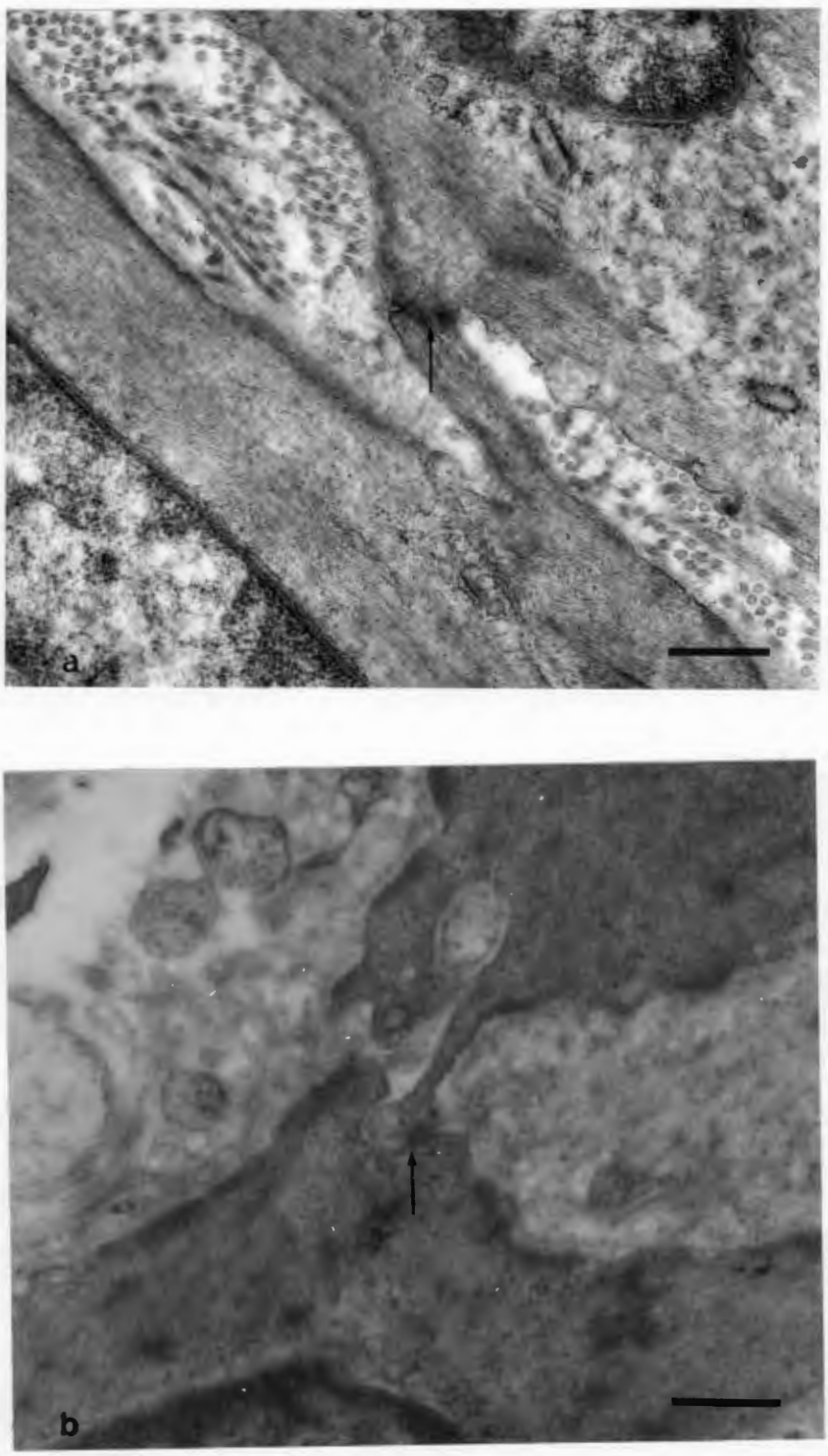

Figure 21: Intercellular connections in the form of gap junctions (arrows) are present in both (a) normal and (b) leiomyomatous myometria. Opposing plasmalemmal densities are often present at these junctions. Scale bar = 0,5μm.

3) MORPHOLOGICAL STUDY TWO

3.1) *Study Aim*

To compare the ultrastructure of normal myometrium and leiomyomata in order to answer the following question: Does the ultrastructure of leiomyomata differ significantly from that of the myometrium from normal uteri?

3.2) *Materials and Methods*

3.2.1) *Patient Sample*

The tissue used for the purposes of this study was derived from the tumours present in the uteri collected from the patients of the leiomyomatous group in morphological study one. The final sample consisted of both large and small leiomyomata from a total of fifty four uteri.

3.2.2) *Methods*

All tissue samples, whether from small or large leiomyomata, were processed for light and electron microscopy in the same manner as the samples in morphological study one. The haematoxylin and eosin stained sections were viewed by brightfield microscopy in order to confirm that each leiomyoma in the sample was of the common type and not one of the histological variants (Chapter 3).

Besides being examined for ultrastructural differences to normal myometrium, all sections in the sample were viewed a number of times in order to assess for any possible effects that the phase of the endometrial cycle and tumour size may have on tumour structure.

3.3) *Results and Discussion*

Superficial ultrastructural examination of leiomyomta reveals that they are remarkably similar to normal myometrium. Thus the following presentation of results and accompanying discussions are limited to those specific ultrastructural features which differ from the normal.

3.3.1) *Phase of Endometrial cycle*

The phase of the endometrial cycle does not appear to significantly affect the ultrastructure of leiomyomata (Figure 22). No evidence of increased myocytic endoplasmic reticulum, free ribosomes or interstitial collagen content are notable in the proliferative phase tumours as a response to increased circulating oestrogen. Likewise, during the secretory phase, when circulating levels of progesterone are relatively high in comparison to oestrogen, little in the way of an increase in the number of myofilaments within the myocytes is discernible.

The similarity between the structure of normal and leiomyomatous tissue, during the various phases of the endometrial cycle, suggests that both forms of tissue respond in a like manner to the normal variations in the levels of the circulating reproductive steroid hormones that occur during the menstrual cycle.

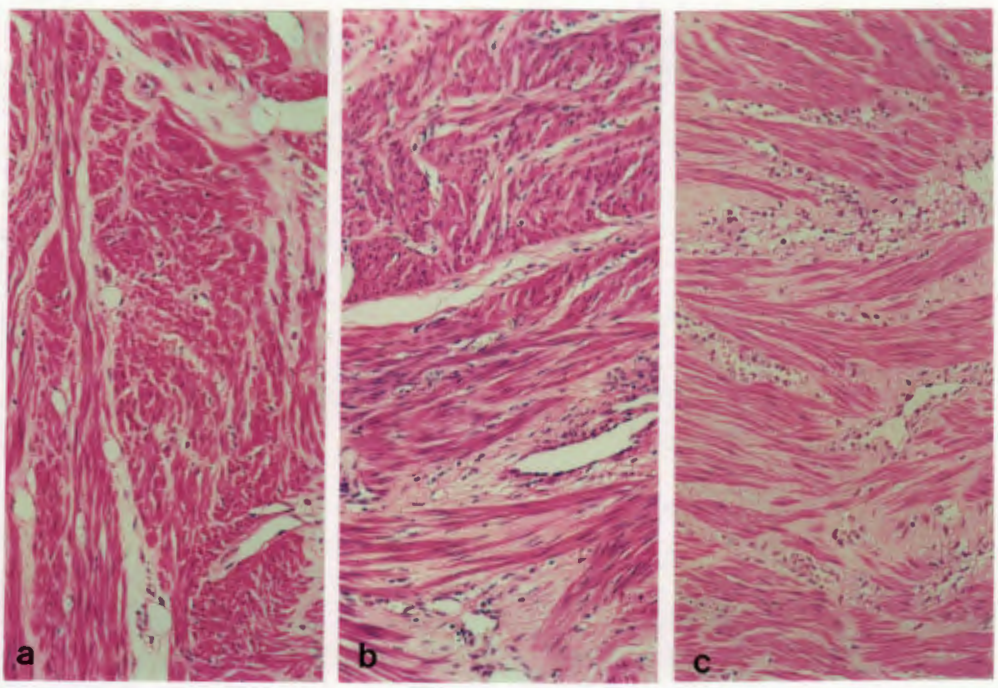

Figure 22: Light micrographs of H&E stained sections of leiomyomata in (a) proliferative (b) secretory and (c) menstrual phase. Print magnification 110x.

3.3.2) *Leiomyomata size and ultrastructure*

Leiomyomata can be arbitrarily classified into minute, small and large. Minute leiomyomata usually measure less than 3mm in diameter, while small leiomyomata range from 3mm to 3cm in size. Any leiomyomata greater than 3cm in diameter may be classified as large. Each of the three categories represents a ten fold increase in tumour size.

The very nature of tissue sampling for electron microscopy limits the total area that can be viewed at any one time. Thus in viewing the majority of sections it is not possible to ascertain by blind analysis whether the sample originates from a large or small tumour. This task is made more difficult by the similarity in myocyte structure and organelle complement between the various samples.

When examined as a group, small fibroids tend to be more myocytic in nature, with less intervening connective tissue, than that of their large counterparts. In comparison, the connective tissue content of very large leiomyomata varies greatly, not only within the individual tumour but between those obtained from the same uterus as well (Figure 23).

Within small leiomyomata, very few myocytes resemble those of the central regions of the minute category, as described by Konishi *et al* (1983). In their electron microscope studies of minute leiomyomata Konishi *et al* (1983) noted, within the central regions of the tumours, irregularly arranged ellipsoid cells with a high nucleocytoplasmic ratio. Abundant free ribosomes and a well developed system of cytoplasmic organelles, in the absence of myofilaments, were characteristic of these cells. The classic plasmalemmal vesicles, that are usually encountered in myocytes, although present, were minimal in number. They also noted that the spindle shaped myocytes of the outer regions of these minute tumours were of a more regular arrangement and were greater in size than those of the central regions. The nucleocytoplasmic ratio of the peripherally located myocytes was markedly reduced with fewer intracytoplasmic organelles. The perinuclear location of the organelles and predominance of myofilaments in these cells makes them virtually indistinguishable from normal myometrial cells.

The observation of two distinct populations of cells within the minute leiomyomata (Konishi *et al* 1983), with their characteristic distributions, is

suggestive of a central nidus of tumour formation and thus lends support to the unicellular theory of origin. It also appears from the morphological characteristics of the small leiomyomata studied, that by the time a ten fold increase in size has taken place, the vast majority of myocytes are of the mature form. It seems likely that the central population of cells in the minute tumours represents a specific group of immature myofibroblasts, that are capable of differentiating into smooth muscle cells given the appropriate stimulus. That very few cells of this character are noted in the small tumours is possibly a reflection of the sampling technique for electron microscopy, where the sampling of the central nidus becomes increasingly less probable as the tumour increases in size.

Although sections from the large fibroids reveal that the majority of myocytes are of the normal mature variety, a small population of unusual myofibrocytic like cells are seen. These large cells have a centrally located nucleus and contain masses of dilated rough endoplasmic reticulum (Figure 24). Mitochondria are large and prominent while blebbing of the plasmalemma is a fairly common feature. The basic structure of these cells is consistent with high levels of protein production. In all cases examined, these unusual cells tend to be surrounded by masses of interstitial collagen and as such they may represent a small, but significant, population of pleuripotential cells, which are capable of switching from the production of myofilaments to collagen in the 'aging' fibroid. The concept of the pleuripotentiality of both mature and immature myocytes has been previously postulated (Willèn *et al* 1978). Sieinski (1989) suggested that such a population of cells may be responsible for the occasional lipomatous differentiation that occurs in some leiomyomata.

If one accepts, from the evidence presented, that leiomyomata are essentially unicellular in origin, then one must accept that the myocyte is a pleuripotential cell capable of both structural and functional metamorphosis. Such changes are reflected in the alteration of the type of protein produced by the cell when subjected to an alternate set of stimuli. Why such a change does not occur in all tumour myocytes and what specific stimuli are needed for this change to occur, cannot be discerned from this study.

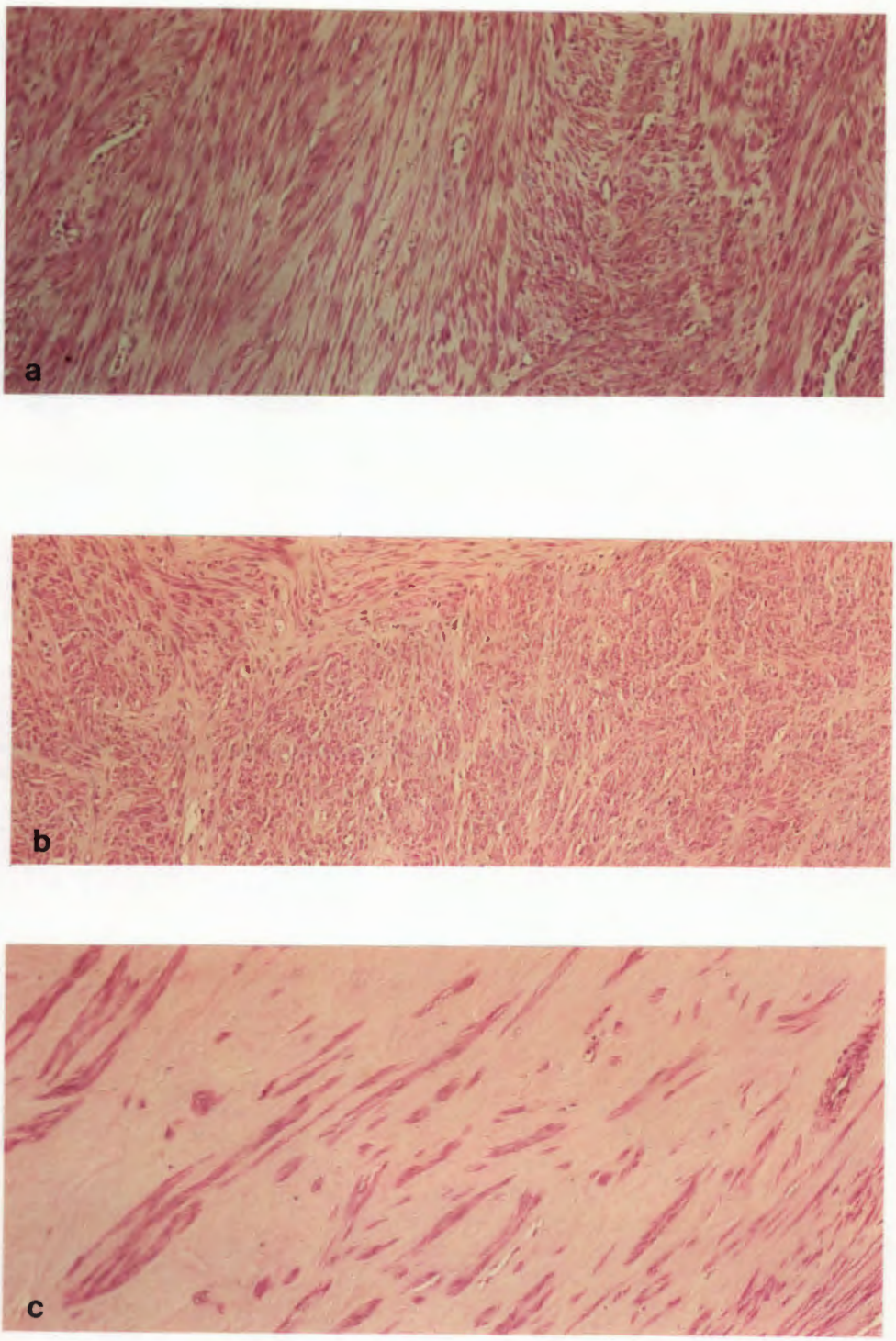

Figure 23: H&E stained sections of (a) small, (b) and (c) large leiomyomata. Print magnification 110x.

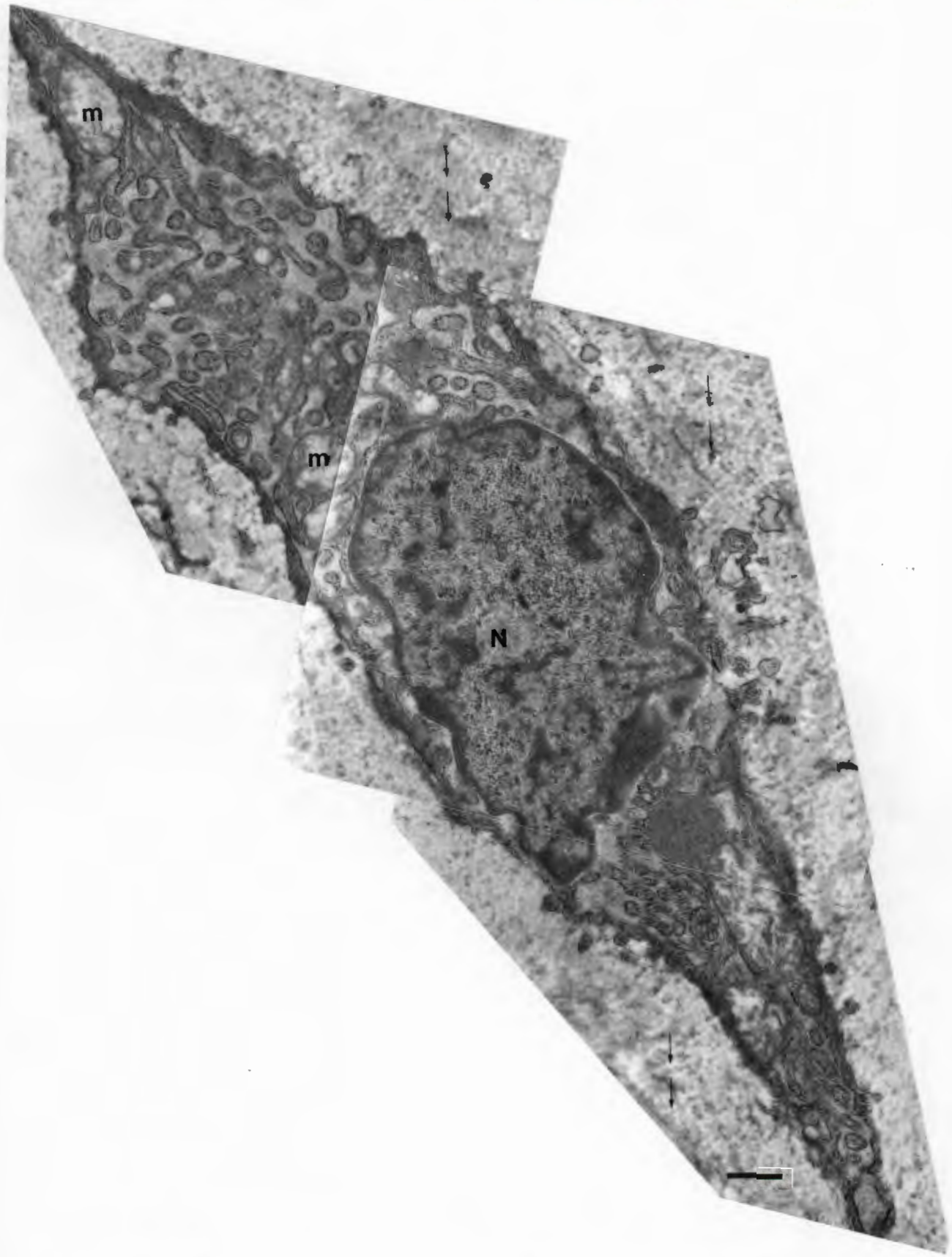

Figure 24: Electron micrograph montage of a myofibrocyte from a large leiomyoma. Multiple intracellular mitochondria (m) and an abundant collagenous interstitium (arrows) are present. N - Nucleus. Scale bar = 0,5μm.

3.3.3) *Specific Ultrastructure*

Nuclear Atypia

Even though nuclear atypia at the light microscopic level is rare, it is encountered, albeit infrequently, with the electron microscope. Usually such atypia is characterised by multiple infoldings of the double nuclear membrane (Figure 25). Occasionally mitochondria and other organelles are trapped within the nuclear folds. It is possible that in some cases this peculiar nuclear appearance may be attributed to the plane of sectioning, however, it is unlikely that this provides an adequate explanation for all the atypical nuclei observed.

No mitotic figures were evident in any of the sections viewed. This does not mean that they do not exist but rather is merely a reflection of how few mitoses there are in these tumours. Tiltman (1985) noted that most leiomyomata demonstrated less than 5 mitoses per 100 high power fields and although this finding is substantiated by the work of Kawaguchi *et al* (1989), they describe an increase in mitotic activity, in secretory phase leiomyomata, with up to 12 mitotic figures per 100 high power fields. In spite of this supposed increase no obvious difference in mitotic activity was detected in the samples studied.

Mitochondria

Although the ultrastructure of the majority of leiomyomatous myocyte mitochondria appears to be essentially normal, regardless of the size of the parent tumour, many of them are qualitatively larger than their normal myometrial counterparts. This increase in size is yet to be assessed and confirmed by morphometric analysis. Not only do the mitochondria appear to be larger but they are also more abundant than those in normal myometrial myocytes (Figure 26). Even though this finding reflects that of Ferenczy *et al* (1971), there remains a large degree of variability within and between tumour samples with regard to mitochondrial number. In a study of thirteen leiomyomata Zukerberg *et al* (1990) noted that 31% of the tumours had sparse mitochondria while the remaining 69% were classed as having a moderate amount of these organelles. They proposed that the amount of mitochondria could be a reflection of the degree of differentiation of the smooth muscle tumour. In the case of leiomyomata this assumption seems unlikely especially when one considers that minute leiomyomata are centrally

packed with mitochondria rich fibromyoblasts. It is more likely that the amount of mitochondria in the sarcoplasm of the myocytes is a reflection of their functional status at the time of fixation.

Myelin bodies, usually in a perinuclear position, are a regular feature of myocytes from large leiomyomata (Figure 27). The presence of such bodies is a classical anatomical feature of cellular injury, most often as a result of ischaemia and hypoxia (Cotran *et al* 1989). Many large leiomyomata do undergo degeneration and although hypoxia is often blamed, many of these tumours appear to have an extensive blood supply (Farrer-Brown *et al* 1970a). In the present study myelin bodies are usually seen in conjunction with varying degrees of endoplasmic reticulum swelling and cellular oedema and are thus possibly indicative of the initiation of the degenerative process.

A number of bizarre forms of mitochondria are also present in leiomyomata but not in normal myometrial myocytes (Figure 28). They are usually characterised by multiple whorls of double membraned lamellae. These structures tend to be more common in the larger leiomyomata. Although their aetiology has not been discerned, abnormalities in the structure of mitochondria may play a role in the decreased contracting power of the uterus in the presence of leiomyomata. This may be due to the abnormal membrane structure interfering with the calcium uptake mechanism of the mitochondrium and thus causing a derangement in the production of action potentials.

Intracellular Filaments

All leiomyomata, whether large or small, contain masses of characteristic myofilaments with a distribution similar to that of normal myometrium. In many of the leiomyomata examined in this study, the relative proportion of intermediate filaments to myofilaments is increased. These 10nm thick intermediate filaments, which usually occupy a perinuclear position where they intermingle with the cellular organelles, fill a substantial portion of the myocytic cytoplasm (Figure 29). As a result, the myofilaments, with their focal densities, are pushed to the periphery of the affected cell. These large aggregates of intermediate filaments are usually well demarcated with the contained filaments arranged in an anisotropic fashion in much the same manner as those described by Eyden *et al* (1992).

Massive aggregates of intermediate filaments, such as those described here, may possibly represent an imbalance between the production and turnover of the filamentous components of the cytoskeleton. This in turn may have a direct bearing on the abnormal contracting power of the leiomyomatous uterus. The specific causes of such an imbalance remain unknown.

Past experimental work has identified the intermediate filaments that are found in normal and leiomyomatous myometria as predominantly desmin and vimentin (Brown *et al* 1987, Evans *et al* 1983, Eyden *et al* 1992, Leoni *et al* 1990, Norton *et al* 1987, Ramaekers *et al* 1988, Turley *et al* 1988). Recent studies of cultured myometrial smooth muscle cells from both pregnant and non-pregnant uterine muscle have revealed that during pregnancy the quantity of intermediate filaments and desmin in particular increase significantly (Leoni *et al* 1990). Vimentin on the other hand has not been shown to undergo any quantitative changes during pregnancy. Results such as these are suggestive of the possibility that only one of the intermediate filament classes may be increased in leiomyomata. Large numbers of intermediate filaments are also associated with developing smooth muscle cells (Uehara *et al* 1971) but as yet they have not been described in association with minute leiomyomata.

Plasmalemmal Densities

Plasmalemmal densities are markedly increased in many of the leiomyomata (Figure 30), much more so than is seen in the leiomyomatous myometrial tissue. Similarly the concurrent decrease in the number of plasmalemmal vesicles is particularly distinct in these tumours. The presence of the plasmalemmal densities does not appear to be affected by the phase of the endometrial cycle or the size of the tumour.

Lipofuscin and Lipid-rich Residual Bodies

When compared to normal myometrial myocytes, from the uteri of patients of comparable age, the smooth muscle cells of leiomyomata exhibit less lipofuscin and far fewer lipid-rich residual bodies. When present, the lipofuscin and lipid-rich residual bodies are of the same structure as those in normal myometrial myocytes and also occupy a paranuclear position (Figure 31).

As the presence of lipid-rich residual bodies has been tentatively linked to ovarian hormone cycling (Yamazaki *et al* 1993), it may be construed, from these results, that the response of leiomyomata to circulating reproductive steroid hormones differs in some way to that of normal myometrium. Alternatively should the lipid from which these bodies are derived originate from the lysosomal breakdown of lipoprotein membranes (Eyden *et al* 1991), then it is equally possible that this mechanism is in some way abnormal in leiomyomata.

The relative lack of lipofuscin in these samples may be attributed to the tumour to host-uterus age ratio, where the tumours are effectively far younger than the host and thus in the age group examined do not show the same degree of aging as the surrounding myometrium in which they are found.

Extracellular Matrix and Fascicular Arrangement

Unlike normal myometrium, the myocytes of leiomyomata are not found in fasciculi with as specific an orientation (Figure 32) as those of their normal counterparts. Cunha *et al* (1989) suggest that the spatial orientation of normal myometrial smooth muscle cells is promoted, during foetal development, by their association with the uterine epithelium. Should this be the case then when one considers that leiomyomata are a post menarcheal event and that they arise most commonly in the wall of the uterus away from possible epithelial influences, then their strange spatial orientation is understandable. In support of this theory is the location of multipotential mesenchymal cells, located in close association with the epithelium, that differentiate into mature smooth muscle cells (Fujii *et al* 1989). However in the case of leiomyomata, even though they probably originate from a similar type of cell which is located within the myometrium, they develop without the epithelial influences experienced by foetal myometrium.

Highly variable amounts of collagen are noted between the groups of leiomyomata myocytes. In spite of this the structure of the collagen does not appear to be ultrastructurally abnormal. Within the extracellular matrix are a variable number of mast cells. Not only do they vary in number but also vary as to structure. Maluf and Gersell (1994) report on finding leiomyomata with more than 10 mast cells per high power field but do not report on the total number of tumours that were surveyed to find these cases. In all of the cases examined no one case appeared to have a higher number of mast cells than the other. Fox and Abell (1965) suggest that leiomyomata may have fewer mast cells than their surrounding myometrium but

due to the intertumour variability in the number of these cells it is not possible, within the confines of this study, to assess the accuracy of this statement. No apparent increase or decrease in mast cell numbers is noted in association with the menstrual cycle.

Finally, in most of the sections viewed capillaries are both present and ultrastructurally normal. It is not possible in most cases to determine whether the vascular structure seen is a lymph vessel or not. Only when erythrocytes were present in the lumen is it possible to make a judgement as to the type of vessel. The extent and spatial arrangement of the vasculature of leiomyomata cannot be made from electron microscope studies due to the limitations of sampling.

3.4) *Conclusion for Morphological Study Two*

Detailed examination of the ultrastructure of leiomyomata reveals that they are not as similar to normal myometria as a first glance would suggest. Many of the abnormalities described have the potential to affect the contracting power of the tissue both directly and indirectly. Also, even though it appears that both normal myometrium and leiomyomata respond structurally in the same manner to the normal variations in serum reproductive steroid hormone levels, it is probable that some form of subcellular derangement exists in the tumorous tissue as evidenced by the lack of lipid-rich residual bodies.

Thus although leiomyomata are histological similar to normal myometrium it can be concluded that they are ultrastructurally distinct entities.

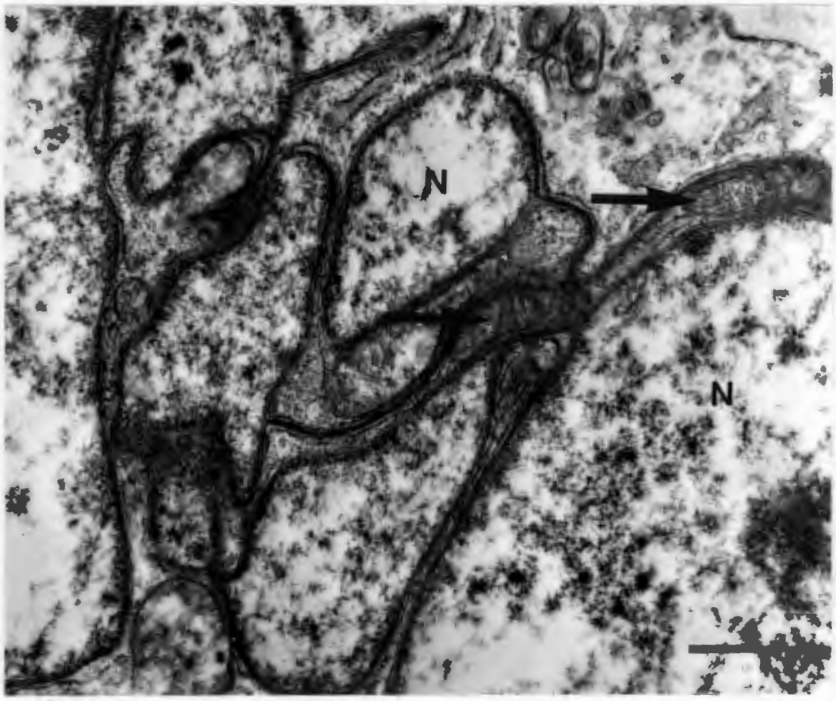

Figure 25: Nucleus (N) from myocyte of large leiomyoma. The nuclear membrane is folded and trapped mitochondria (arrows) can be seen. Scale bar = 0,5μm.

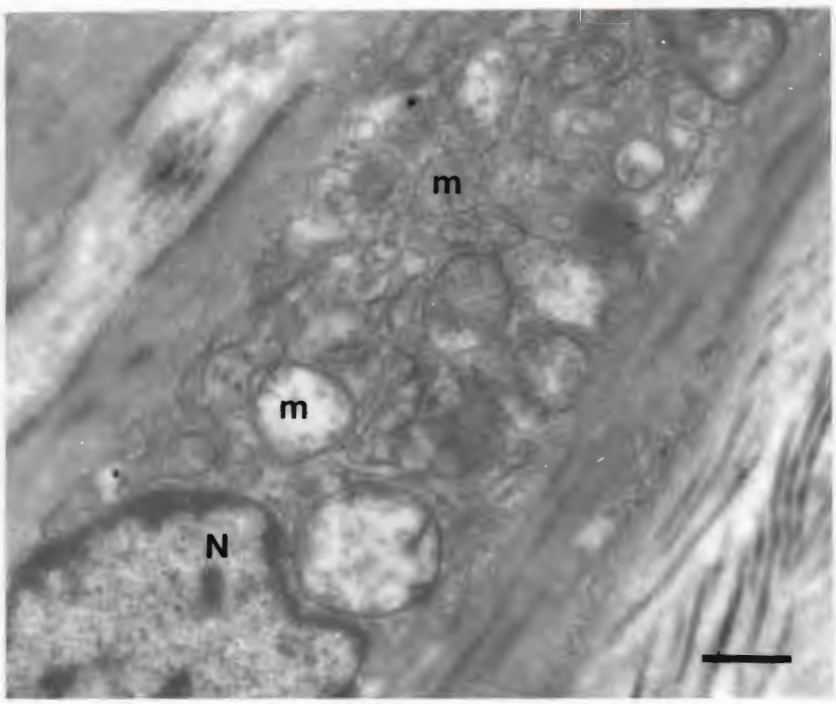

Figure 26: Leiomyomatous myocyte with perinuclear organelles. Multiple large mitochondria (m) fill most of the sarcoplasm. N - Nucleus. Scale bar = 0,5μm.

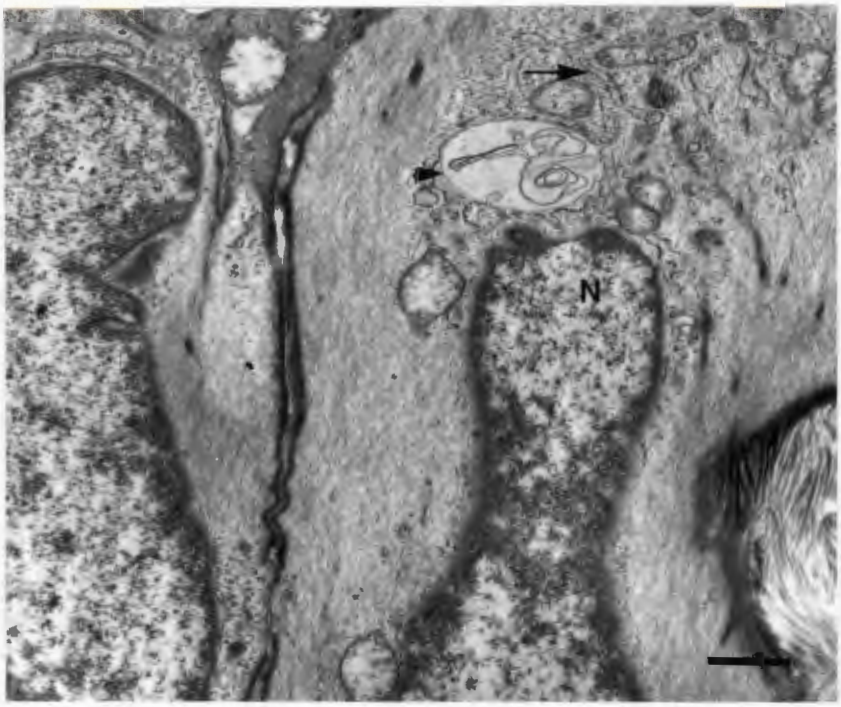

Figure 27: Electron micrograph from a leiomyoma demonstrating a perinuclear myelin body (arrowhead) and dilated endoplasmic reticulum (arrow). N - Nucleus. Scale bar = 1μm.

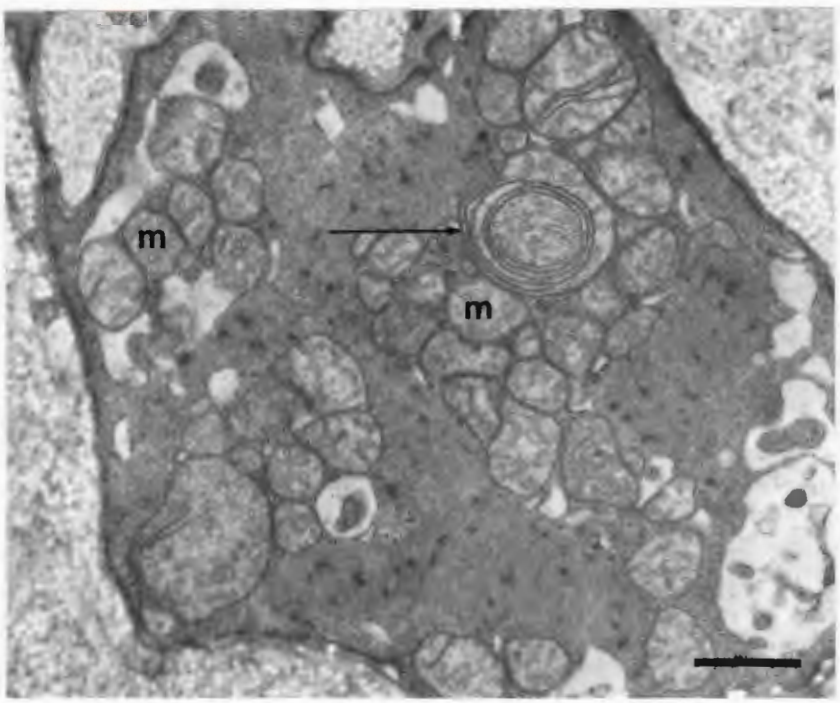

Figure 28: Leiomyoma myocyte with multiple mitochondria (m) as well as a bizarre whorled mitochondrium (arrow). Scale bar = 1μm.

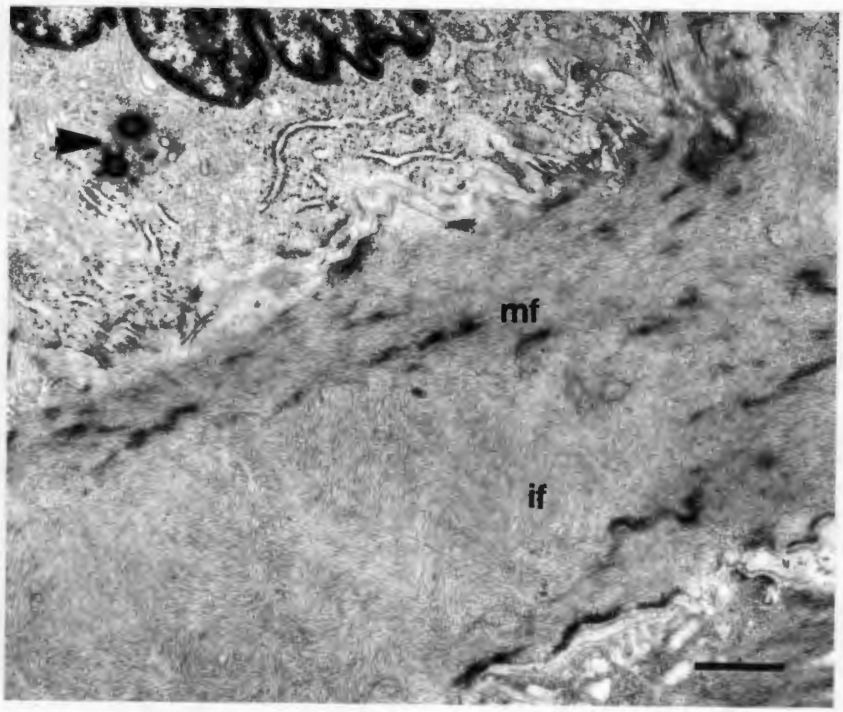

Figure 29: Electron micrograph of a smooth muscle cell from a large leiomyoma demonstrating masses of wavy intermediate filaments (if). Myofilaments (mf) are located at the periphery of the intermediate filament aggregate. A centriole is also seen (arrowhead). Scale bar = 1μm.

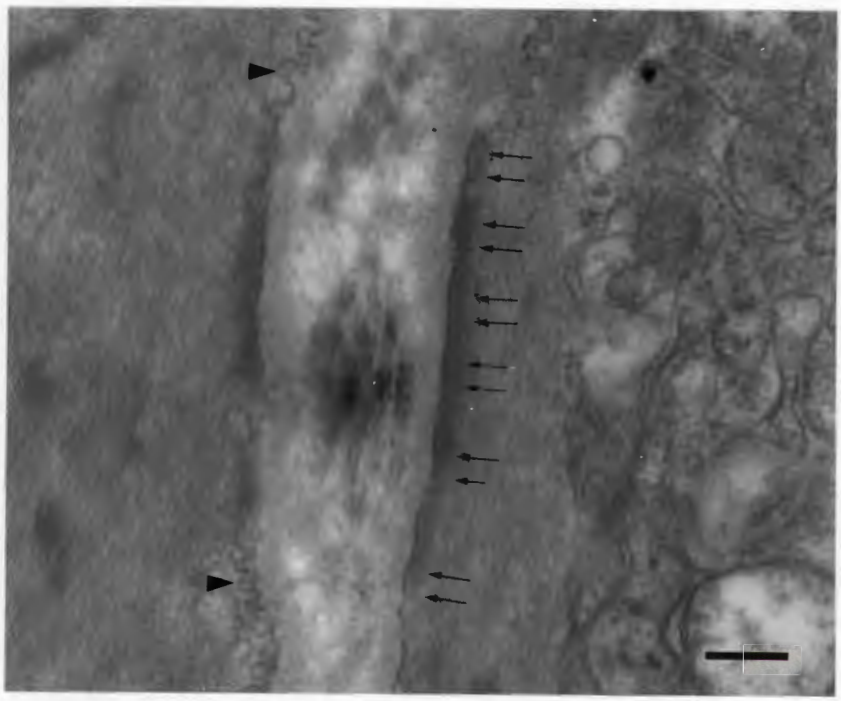

Figure 30: Extensive plasmalemmal densities (arrows) and relatively few plasmalemmal vesicles (arrowheads) are a common feature of leiomyomata. Scale bar = 0,25μm.

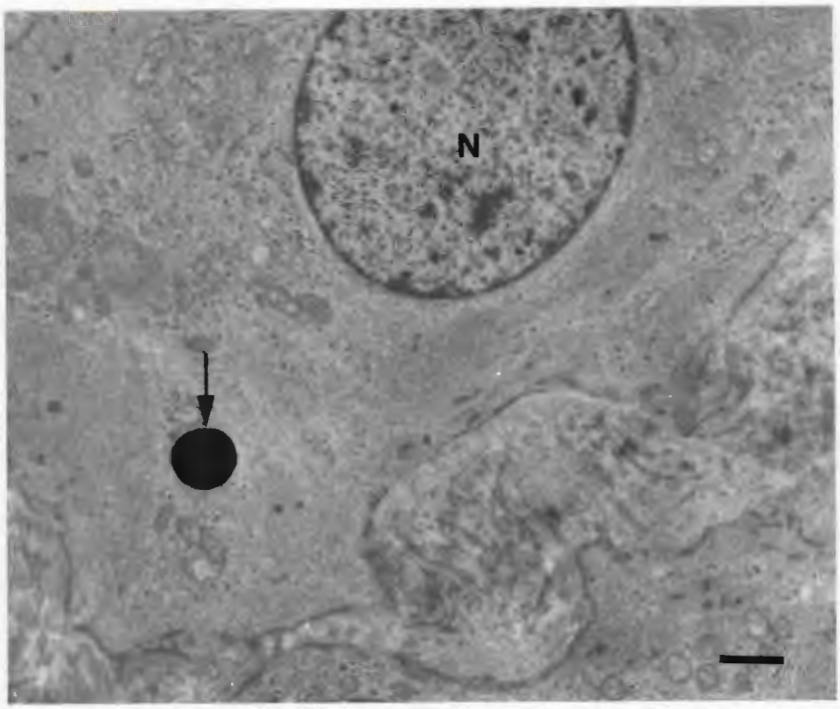

Figure 31: Lipid-rich residual bodies (arrow) are found in association with the perinuclear organelles of leiomyomatous cells. N - Nucleus. Scale bar $=1\mu$m.

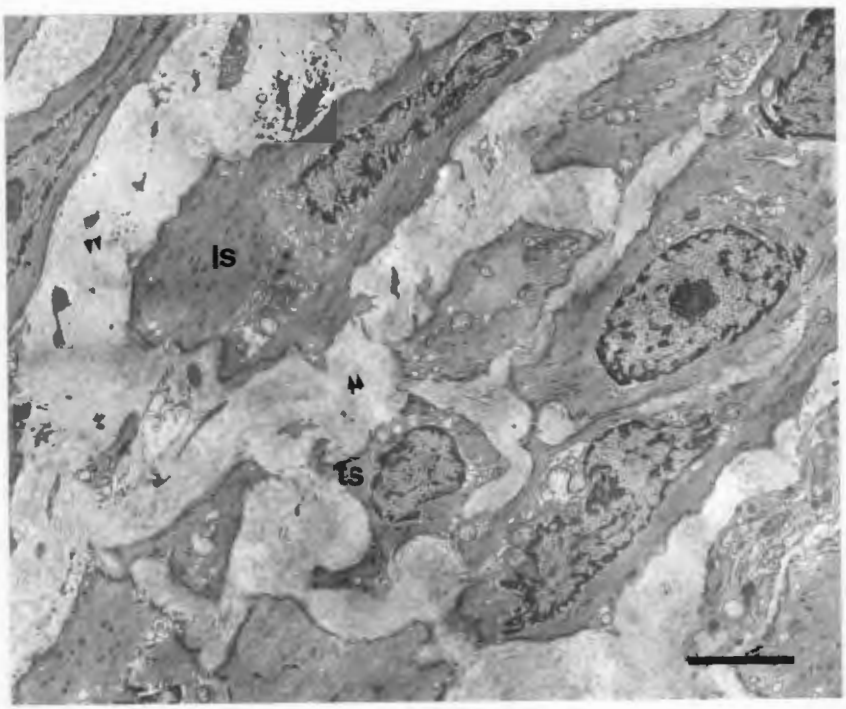

Figure 32: Electron micrograph of a section of a leiomyoma in which myocytes are seen cut in both transverse (ts) and longitudinal section (ls) within an extenstive collagenous matrix (arrowheads). Scale bar $= 5\mu$m.

4) MORPHOLOGICAL STUDY THREE

4.1) *Study Aim*

To assess whether normal myometrium and leiomyomata respond, ultrastructurally, in a like manner to increasing age and parity.

4.2) *Materials and Methods*

4.2.1) *Patient Sample*

The patient sample for this study was divided into two broad categories so that age and parity could be assessed separately.

Firstly, in order to assess the effects of aging, on the ultrastructure of myometrium and leiomyomata, a series of twelve normal and twelve leiomyomatous uteri were examined. The uteri chosen for this study were obtained from patients with no history of exogenous reproductive steroid hormone ingestion and ranged in age from 13 years to 71 years. To avoid any possible influence that high parity may have on the ultrastructure of myometrium and its leiomyomata, only patients with a parity of three or less were selected.

Secondly, in a similar fashion, a group of twelve normal and twelve abnormal uteri were selected for the assessment of the ultrastructural effects of increasing parity. Parity for the selected sample ranged from 0 to 13. For this particular part of the study, the sample was extracted from the 31 to 70 year age groups.

4.2.2) *Methods*

Tissue samples of leiomyomata, normal and leiomyomatous myometrium, were collected and processed in the same manner, for both the age and parity components of this study, as in morphological studies one and two. Blind analysis, using the electron microscope, was carried out in order to eliminate, as far as possible, operator bias with regard to the description of structure.

4.3) *Results and Discussion*

4.3.1) *Age effects in Normal and Leiomyomatous Myometria*

In both normal and leiomyomatous myometria the most prominent ultrastructural feature, associated with increasing age, is the alteration in the quantity of lipid-rich residual bodies and lipofuscin. In those samples of tissue extracted from uteri of less than 20 years in age, only small amounts of lipid-rich residual bodies are present (Figure 33), while lipofuscin is absent. Myocytes from uteri in the middle-aged group demonstrate both lipid-rich residual bodies and lipofuscin in varying proportions. A predominance of lipofuscin is seen in the smooth muscle cells of the post-climacteric uteri with minimal to no lipid-rich residual bodies (Figure 33).

The relative increase in the quantity of lipid-rich residual bodies, in relation to the onset of menarche, with their predominance during the reproductive years and decline in the post-climacteric, supports the theory of their presence being influenced by the levels of circulating reproductive steroid hormones (Eyden *et al* 1991). Thus, as reproductive hormone levels increase with the onset of adolescence, so do the number of lipid-rich residual bodies. Likewise, declining reproductive hormone levels, after menopause, are reflected by a decrease in the numbers of these structures.

The presence of lipofuscin is commonly associated with the degradative processes that accompany aging and although their presence in the uterus has been documented, usually in association with conditions such as Friedreich's ataxia (Siboni *et al* 1987), there is little information regarding lipofuscin in the aged uterus. The increase in the appearance of lipofuscin in the aging uterus, as seen in this study, may be attributed to not only the normal aging process but also to the maturation of lipid-rich residual bodies. The actual mechanism of this change is uncertain but may be related to the original process whereby lipid-rich residual bodies are formed in response to the cyclical stimulation of lipid synthesis by oestrogen and with time these bodies mature into lipofuscin. This would explain why there is a decrease in lipid-rich residual bodies and an increase in lipofuscin in the older samples when the oestrogen stimulus is removed.

For both the normal and leiomyomatous myometria, it is not possible, using the electron microscope, to positively associate aging and the quantity of interstitial

collagen. This is due to the great variation in collagen content both within and between samples. One might have expected to find a higher muscle to collagen ratio in the younger uteri but as can be seen from Figure 34 this is not always the case. Dilts and Greene (1964) were also not able to find, at the light microscope level, an increase in myometrial collagen in response to age. In the older uteri smooth muscle cell atrophy was fairly common (Hendrickson & Kempson 1992).

Hendrickson and Kempson (1992) report that the uterine arteries undergo a disproportionately high degree of degeneration, in the form of intimal proliferation, fibrosis and medial calcification, as they age. The small calibre vessels examined in this study do not show the same degenerative trend.

4.3.2) *Age Effects in Leiomyomata*

Leiomyomata present a singular problem when the effects of aging are studied. It is clear that as the tumours do not develop in conjunction with the foetal myometrium, dating the tumours in respect of their host myometrium is impossible. It is, at present, also not possible to establish whether normal myometrium and leiomyomata age at the same rate. Should these tumours be unicellular in origin, it follows that the larger they are, the 'older' they are. The variations in structure in relation to size have been discussed in morphological study two. The evidence that both lipid-rich residual bodies and lipofuscin are decreased in quantity in leiomyomata also suggests that they may not be as metabolically mature as their host myometrium.

In terms of interstitial collagen content; in general, the larger the fibroid, the greater the content of collagen. Collagenisation of the 'aging' fibroid is most likely brought about by the presence of a population of pleuripotential myofibrocytes.

4.3.2) *The Effects of Increasing Parity*

In response to repeated hypertrophy of the myometrium, the overall weight and thickness of the uterine wall increases in conjunction with increasing parity (Hendrickson & Kempson 1992). There has been much debate as to whether or not the collagen content of the myometrium also increases with parity. Woessner (1963) reports that the collagen content, at the light microscope level, of the nulliparous uterus is far less than that in the multiparous uterus, however, Dilts and Greene (1964) report precisely the opposite.

In the present sample the variation in the quantity of interstitial collagen is so great within and between specimens of different parity, that it is not possible to accurately assess its distribution. Likewise quantification of the collagen content of these samples at the electron microscope level is impossible.

Furthermore the accurate assessment of the ultrastructural effects of increasing parity on both normal and leiomyomatous tissue is made more difficult by the age range that this sample covers. This is because the sample itself reflects the changing attitudes to family size within our local society. The availability of high parity samples is limited to the older generation where contraceptive practices were not as readily available or used, whereas the younger sample tends to have very few instances of high parity. Thus any differences would have to be examined in light of the age discrepancy of the sample, however no structural differences of note are seen.

No variation in the ultrastructure of the myocytes or the collagen content of leiomyomata from patients over the whole range of parity can be discerned.

4.4) *Conclusion for Morphological Study Three*

In terms of the conventional annular aging process, normal myometria and leiomyomata do not respond, ultrastructurally, in the same way. This may be attributed to their differing times of origin as well as to their different growth patterns.

The response of normal and tumorous tissue to the effects of increasing parity cannot be accurately assessed in this sample. To do so would require a larger sample isolated in terms of culture, age and parity.

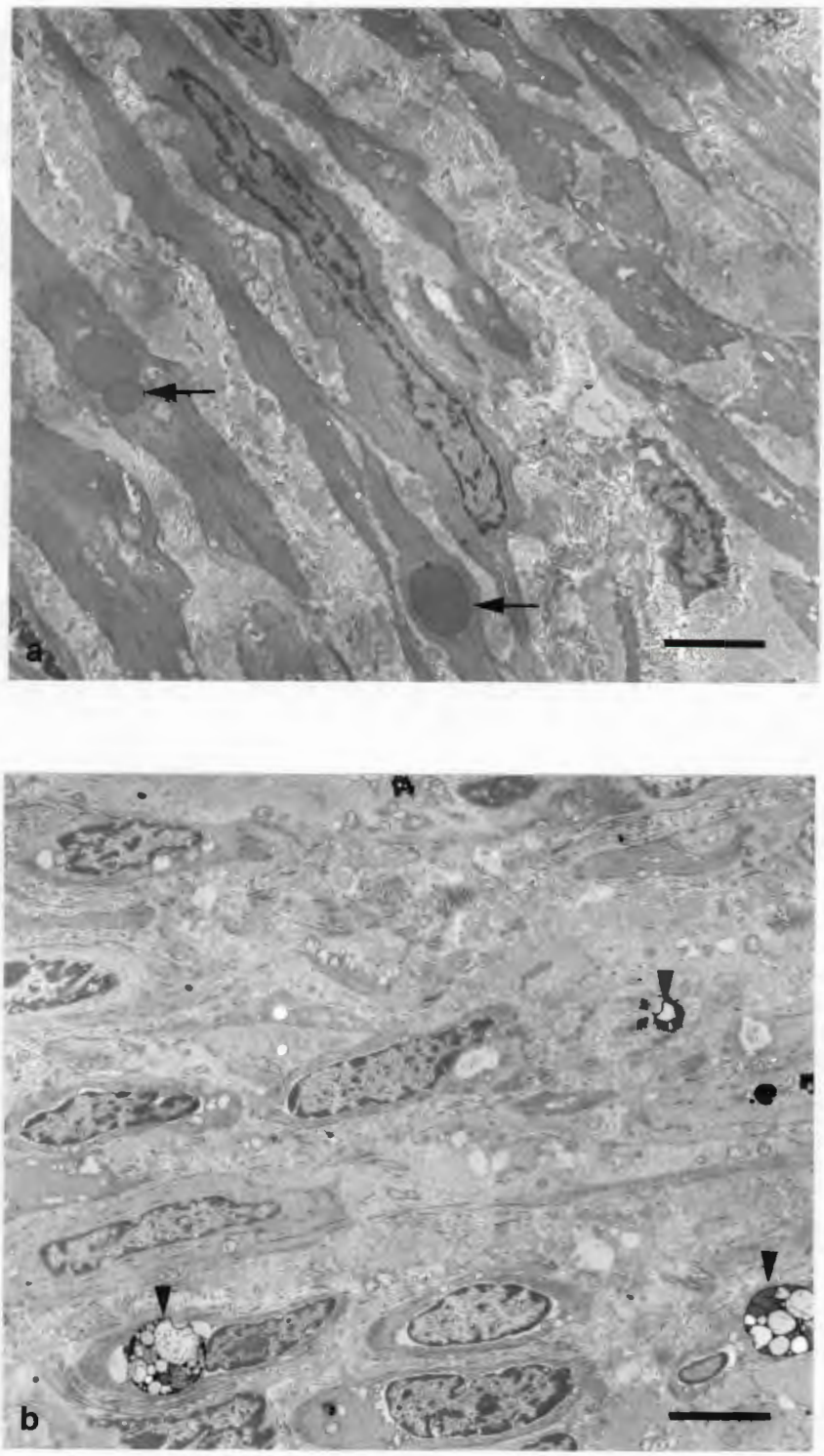

Figure 33 : Electron micrographs of myometrium (a) at menarche and (b) post-climacteric. Note the relative proportions of lipid-rich residual bodies (arrows) and lipofuscin (arrowheads). Scale bar = 5μm.

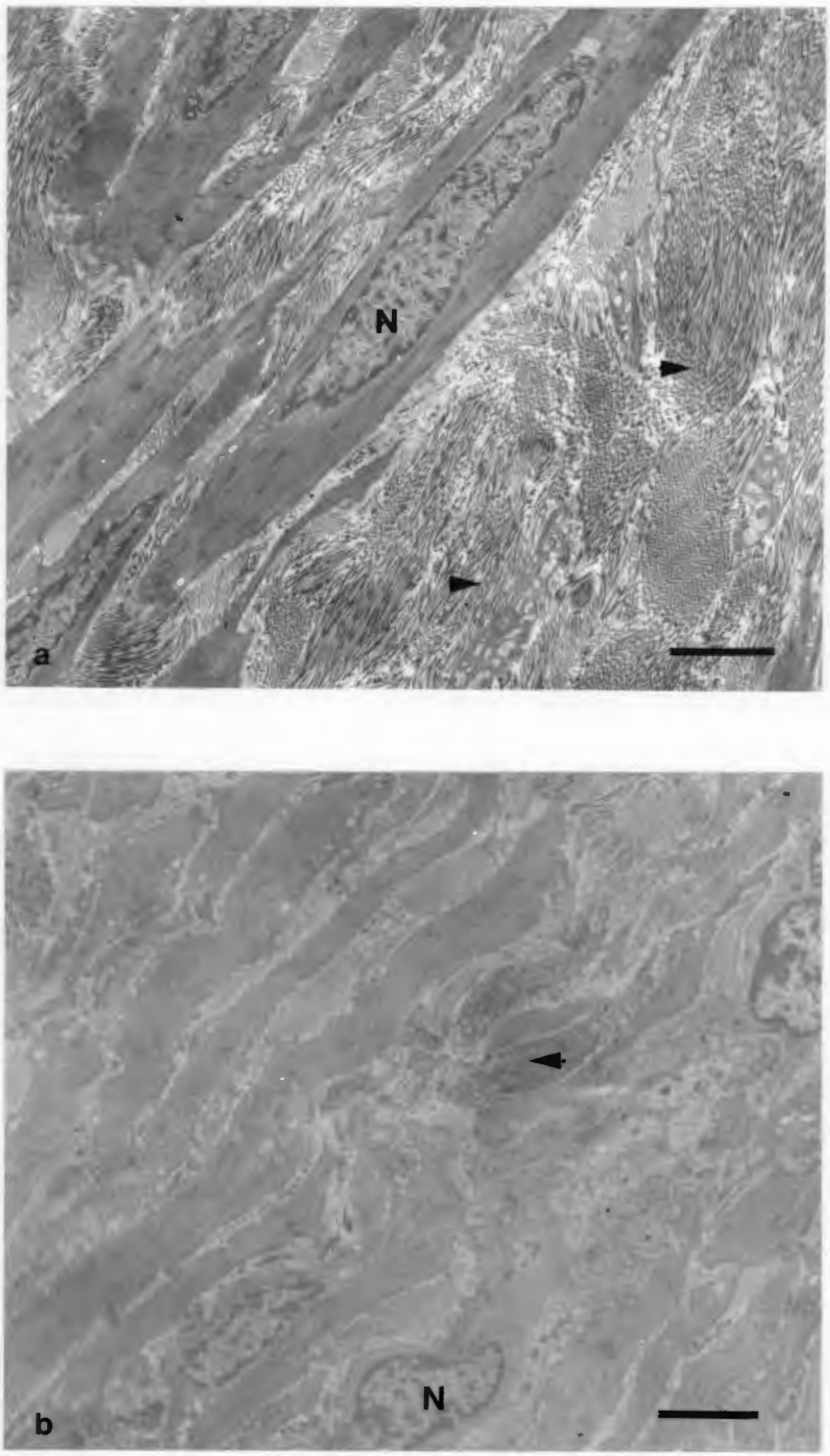

Figure 34 : Elecron micrographs of myometrium (a) at menarche and (b) post-climacteric. Marked variation in the quantity of interstitial collagen is seen (arrowheads). N - Nucleus. Scale bar = 5μm.

CHAPTER EIGHT

IMMUMOCYTOCHEMICAL LOCALISATION OF THE OESTROGEN RECEPTOR IN NORMAL AND LEIOMYOMATOUS TISSUE

1) STUDY RATIONALE

As outlined in chapter five the underlying purpose of this thesis is to gain an understanding of the fundamental character of myometrium and its leiomyomata. In the previous chapter a group of morphological studies, aimed at gaining such an understanding at the electron microscope level, were presented. In this chapter experimental work is detailed, using predominantly immunocytochemistry, in order to answer the three basic questions of the thesis at a more 'functional' level.

For the requirements of this thesis, the experimental work was restricted, for a number of reasons, to the light microscopic localisation of the oestrogen receptor. Firstly, as oestrogen is the reproductive steroid hormone that is most commonly implicated in the causation of leiomyomata, further information on the distribution and quantity of the oestrogen specific receptor could prove invaluable in understanding the nature of normal and tumorous tissue. Secondly, the current question to be addressed is whether or not normal and neoplastic myometria respond in a similar fashion to an external stimulus and not the effect of the different reproductive steroid hormones on the tissue. Thirdly, rationalisation was essential due to the logistics of the following studies. Finally, although monoclonal antibodies for the localisation of the progesterone receptor in paraffin embedded tissue are available, they have not been proven, within this institution, to be sufficiently accurate on myometrial tissue for the requirements of the cellular quantification that was undertaken.

2) AN INTRODUCTION TO OESTROGEN RECEPTOR STUDIES

In recent years the accurate localisation and quantification of various hormone specific receptors has become of paramount importance for the identification and prognostic assessment of many disease states (Ehrlich *et al* 1981, Sumida *et al* 1985). Groups of hormone specific proteins or receptors that preferentially bind the reproductive steroid hormone oestrogen (Gabb & Stone 1974, Krishnan *et al* 1973, Pollow *et al* 1978) have been isolated in both breast and uterine tissue (Notides *et al* 1972, Walters 1985). The interaction between oestrogen and its specific receptor produces a conformational change at the mid-receptor region, unmasking high affinity DNA binding sites with subsequent DNA activation (Gorski *et al* 1986) and ultimately, the synthesis of new proteins. Thus, under the influence of oestrogen, increases in uterine size and endometrial proliferation are achieved (Anderson *et al* 1975). This oestrogen-receptor complex interaction also produces an increased affinity for subsequent oestrogen binding, as well as the activation of progesterone specific receptor production (Speroff *et al* 1989).

Multiple studies involving the reproductive steroid receptor for oestrogen have been conducted, using uterine tissue, in an attempt to elucidate the relationships between the phase of the endometrial cycle, the level of circulating oestrogen and the quantities of the myometrial oestrogen receptor (Runnebaum *et al* 1978, Schmidt-Gollwitzer *et al* 1979). Variations between the receptor levels of normal and diseased uterine tissue have also been investigated (Farber *et al* 1972, Marugo *et al* 1989, Okulicz *et al* 1990, Soules & McCarty 1982, Tamaya *et al* 1979, Wilson *et al* 1980).

In most of the studies involving myometrial tissue it has been generally accepted that the oestrogen receptor is uniformly distributed throughout the depth of the normal myometrium. When dealing with leiomyomatous uteri the basic assumption has been made that the myometrium in which the tumours occur is normal with regard to the quantity and distribution of the receptor. The following studies have been carried out in order to assess, firstly, whether these basic assumptions hold true and secondly, whether the oestrogen receptor content of leiomyomata differs to that of normal myometrium.

3) IMMUNOCYTOCHEMICAL STUDY ONE

3.1) *Study Aim*

To investigate whether there is variation of the oestrogen receptor content of different areas of the normal uterus during both the proliferative and secretory phases, in order to standardise subsequent myometrial control samples.

3.2) *Materials and Methods*

3.2.1) *Patient Sample*

A total of fifty uteri from patients in the 31 to 50 year old age group, with no history of exogenous hormone intake, were selected for this study. Of these uteri thirty were in the proliferative phase of the endometrial cycle while the remaining twenty were in the secretory phase. In both groups of uteri there were five blocks of lower uterine segment that were unsuitable for study purposes as their endometrium had not been included.

3.2.2) *Methods*

Using the tissue collected and stored, as described in chapter five, processing was carried out as follows: 1μm thick wax sections were cut, stained with haematoxylin and eosin (Appendix I:IV) and viewed by standard brightfield microscopy in order to confirm histologically both the tissue normality and the endometrial cycle phase.

The oestrogen receptor content of the myometrium of six randomly selected samples, from the proliferative phase, was determined by radioimmunoassay (RIA) using the single saturation point assay method (Marugo *et al* 1987) (Appendix I:VII). A total of twenty and thirty samples, from the varying areas and regions respectively, from the six patients, were analysed. The tritiated steroid [2,4,6,7 ^{3}H] oestradiol (102 Ci/mmol), obtained from Amersham International Laboratories was used at a final concentration of 6nmol. The concentration of oestradiol receptors was measured by an exchange procedure where the unbound hormone was removed using dextran coated charcoal (Farber *et al* 1972). The binding data were analysed by Scatchard analysis (Scatchard 1949) and expressed as fmol/mg of cytosolic

protein. The cytosolic protein was quantified by the method of Lowry et al (1951) using bovine serum albumin as standard (Appendix I:VIII).

Immunocytochemical staining for oestrogen receptors in the paraffin embedded tissue was performed by the avidin-biotin technique. The technique described in detail in Appendix I:IX may be summarised as follows: Blocked dewaxed sections were microwaved, in citrate buffer (pH 6,0), in a 600 watt household microwave oven at 75% power for two 10 minute periods, in order to effect antigen retrieval. Sections were successively treated with 1) normal rabbit serum (1:20 dilution), followed by 2) the monoclonal oestrogen receptor antibody (Dako-ER M7047 1:50 dilution) and then 3) a biotinylated rabbit anti-mouse was applied. 4) Streptavidin at a 1:500 dilution was applied prior to diaminobenzidine-H_2O_2 (DAB). Each antibody application was followed by a three minute wash in TRIS buffer (0,001 M tris-HCL, 1,5mM ethylenediaminetetraacetic acid, pH 7,4). The sections were then lightly counterstained with haematoxylin, dehydrated and mounted.

Sections were viewed and photographed by Hoffman Modulation Contrast microscopy. The counting of immunopositive cells within a given area is a routine quantitative procedure where the validity of the results is usually dependant on the contrast and intensity of the positively stained cells. However, accurate quantification utilising brightfield microscopy usually presents a number of problems. These include false negatives due to the failure of recognition of positive cells of low contrast and the poor repeatability of operator dependant optical systems. Hoffman Modulatiom Contrast microscopy improves contrast and eliminates differences in optical intensity in the sections and thus increases the potential for obtaining accuracy in the quantification of positive cells. For Hoffman Modulation Contrast microscopy the light microscope is set up for Köhler illumination in the same fashion as for bright field microscopy (Figure 35). In place of the illuminating aperture diaphragm is a strip aperture which is aligned with the modulator plate in the back focal plane. The modulator plate consists of an opaque area, an area of neutral density and clear glass (Hoffman & Gross 1975). As a result, topographic features are unilaterally highlighted on a grey background producing a three dimensional image. Using this form of microscopy Richards *et al* (1994) noted improved intensification of immunopositive cells due to the greater surface irregularities seen on those nuclei where the DAB reaction product was present.

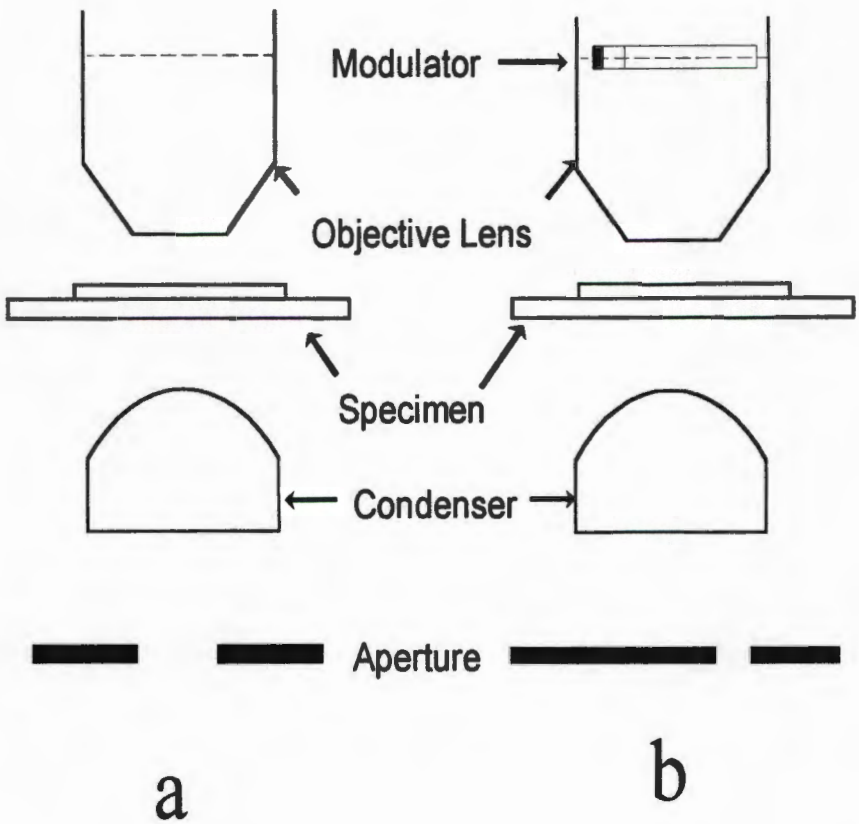

Figure 35: The optical components for (a) Brightfield Microscopy and (b) Hoffman Modulation Contrast Microscopy

For counting purposes, every slide, whether from the fundus or lower segment, was subdivided into subendometrial, midmyometrial and subserosal regions. The cell populations of five high power fields per region, per slide, were counted twice for each case. Any cell showing evidence of the DAB reaction product was considered to be positive. A further 100 positive staining nuclei per region per slide were examined for the percentage of nuclear staining and were divided into three catergories. These being A) staining of less than one third of the nucleus, B) between one and two thirds and C) between two thirds and the entire nucleus.

3.3) *Results*

Brightfield microscopy of the haematoxylin and eosin stained sections confirmed that the dissected blocks of tissue were taken from the fundal and lower segment regions, that all uteri were in either the proliferative or secretory phases and that the tissue was histologically normal.

Table 6: Myometrial concentration of oestradiol receptors as determined by Radioimmunoassay (fmol/mg)

Patient	SS n=20	M n=20	SE* n=20	Fundus n=30	Lower segment** n=30
1	8,03	13,56	25,69	15,70	15,81
2	4,63	10,50	11,92	8,51	10,84
3	13,03	16,43	44,43	25,71	23,55
4	10,08	10,50	43,51	22,41	20,58
5	5,59	6,86	20,98	12,63	9,65
6	15,63	26,19	43,29	32,57	24,16
Mean	9.5	14.01	31.54	19.59	17.43
Std.Dev.	13.99	6.78	4.28	8.95	6.31
SEM	3.13	1.52	0.96	1.63	1.15

SS - Subserosal, M - Midmyometrial, SE - Subendometrial regions.

* Subendometrial region significantly different from subserosal and middle regions ($p < 0,01$).

** No significant difference between Fundus and Lower segment regions.

The results of the radioimmunoassay for the oestrogen receptor content of the normal myometria are presented in Table 6. Using a students T-test (Allan 1982), the oestrogen receptor content of the subendometrial portion of the myometrium is shown to be statistically significantly greater than that of the middle and subserosal areas ($p<0,01$). The receptor content of the fundal and lower uterine segments is also presented in Table 6. There is no significant difference discernible between the two segments.

In immunocytochemically positive cells the DAB reaction product is limited to the nucleus, with no evidence of cytoplasmic or background staining. The nuclei of the uterine blood vessels do not stain for the oestrogen receptor. Variations in the myometrial cellularity and positivity, expressed as total cells per high power field, are presented in Tables 7 and 8. Of the three areas, the subendometrium proves to be the most cellular, with an average of 106 nuclei counted per high power field. Statistical testing, using the analysis of variance test, shows that highly significant differences exist between the numbers of immunopositive cells in the three transmural areas, where $p < 0,0001$. The same test does not reveal any significant difference between the values obtained for the fundal region and lower uterine segment ($p < 0,6502$). Likewise the phase of the menstrual cycle does not effect positivity and no significant diffence is noted between the positive counts for the proliferative and secretory phase myometria respectively ($p < 0,1503$)(Figure 36).

Analysis of variance testing also fails to reveal any significant interactions between area and region ($p < 0,1186$), region and phase of menstrual cycle ($p < 0,4576$), or between area and phase of cycle ($p < 0,5985$). Almost 83% of all cells counted in the subendometrial region are positive, while only 61% and 47% of cells in the midmyometrial and subserosal regions respectively, demonstrate positivity.

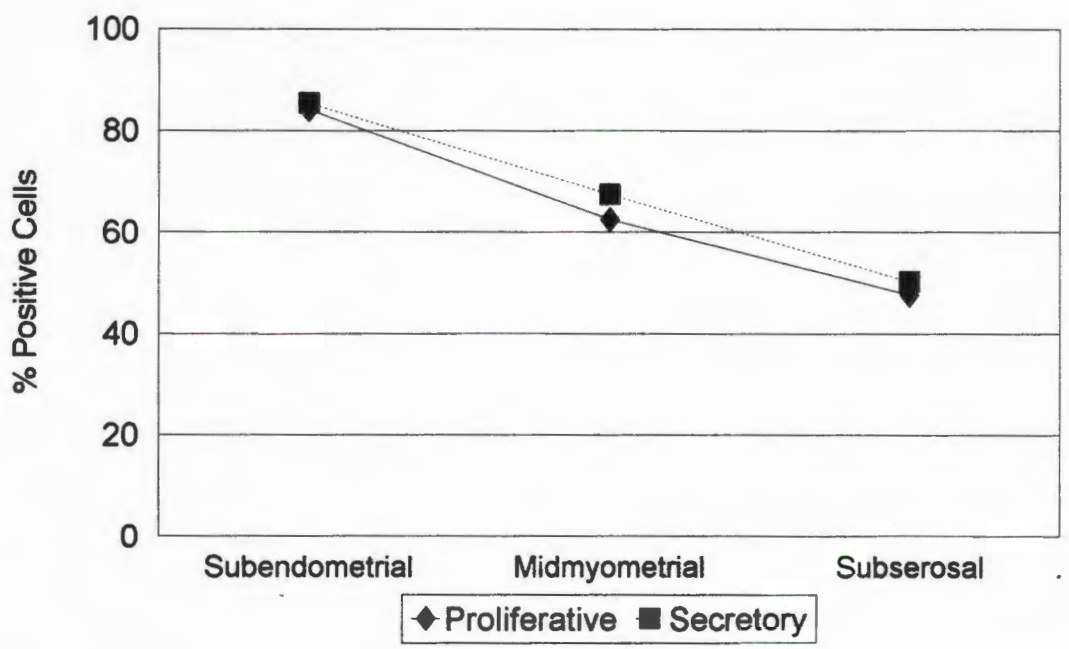

Figure 36: Graphical representation of the oestrogen receptor content of myometrium during the proliferative and secretory phases of the menstrual cycle.

The percentage nuclear staining in samples of proliferative and secretory myometrium are presented in Tables 9 and 10. Nuclear staining varies greatly, with the entire nucleus often obscured by reaction product in the subendometrial area while in the midmyometrial and subserosal areas nuclear staining is commonly unilateral or circumferential (Figure 37). Quantitative assessment of the percentage nuclear staining reveals that over 60% of nuclei within the subendometrial region are greater than two thirds stained, while in the subserosal region an almost equivalent number of nuclei are less than one third stained (Tables 9 and 10).

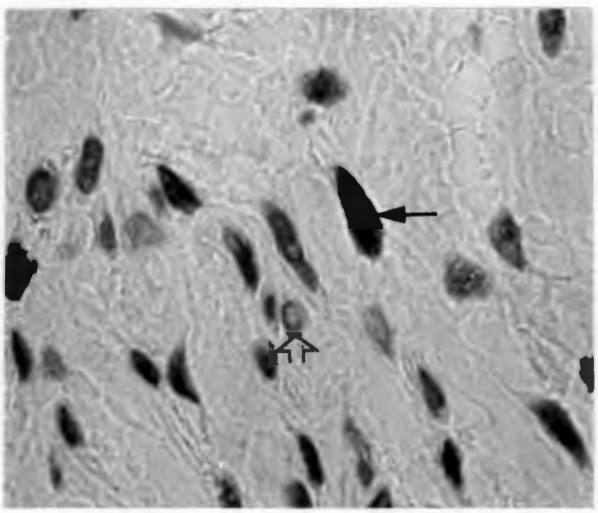

Figure 37: Nuclear staining for the oestrogen receptor in the midmyometrial region. Some nuclei are completely obscured by reaction product (arrow) while others are only circumferentially stained (open arrow). Print magnification x1280.

3.4) *Discussion*

3.4.1) *The Value of Radioimmunoassay*

In recent years the biochemistry of the oestrogen receptor has been more clearly elucidated (Gorski *et al* 1986, McCarty et al 1985). It is now known that the receptor is a purely nuclear resident which is only loosely bound to the nucleus when in the unoccupied state (King & Greene 1984, Press & Greene 1988, Welshons *et al* 1984). Any disruption of the cell, such as that produced by RIA techniques, causes the oestrogen receptor to leak out into the cytoplasm. The cytosolic receptor fraction measured by RIA is thus an extraction artifact and the value of such figures is of minimal use in the determination of total actual quantities of oestrogen receptors.

However, the figures can be useful when investigating trends, provided that all the samples are treated in precisely the same manner and are quantified at the same time. As evidenced from the results obtained in the RIA component of this study, a difference does exist, in which there is an increase in the oestrogen receptor content of the subendometrial region in comparison to the subserosa. These results and the nuclear nature of the receptor may provide, in part, an explanation for the large standard deviations obtained by previous researchers of myometrial oestrogen receptor content (Marugo *et al* 1989, Runnebaum *et al* 1978).

3.4.2) *Transmural Oestrogen Receptor Gradient*

Immunocytochemistry, in comparison to RIA, provides an accurate visual method for the localisation of the oestrogen receptor (Press *et al* 1986). As noted by other researchers, the oestrogen receptor is restricted to the nucleus of positive staining cells (King & Greene 1984, Press & Greene 1988, Welshons *et al* 1984). Tsibris *et al* (1981) suggested that a steep concentration gradient in the oestrogen receptor levels, from the fundal to the cervical regions of the human uterus, exists and that this gradient persists throughout the menstrual cycle. In this study and similar to that of Press and Greene (1988) no such gradient was demonstrated. However, from the immunological cell counts obtained for positivity, it appears as if differences in the concentration gradient of the oestrogen receptor do exist transmurally in the normal myometrium.

The mechanism whereby such a marked transmural concentration gradient occurs, with respect to oestrogen receptor quantity, is unclear. However, as the subendometrial portion is markedly richer in receptors, than the outlying areas, it may well be that distance from the oestrogen sensitive endometrium influences receptor production. Alternatively, the normal mature myometrium may be differentially sensitive to circulating oestrogen, thus producing this specific pattern of receptor distribution.

3.4.3) *The Endometrial Cycle and Receptor Quantity*

The marked differences in the receptor concentrations obtained by different groups of researchers (Marugo *et al* 1989, Vihko *et al* 1980) makes comparisons almost impossible. Few researchers agree as to whether or not the oestrogen receptor content of the myometrium is affected by the normal variations in the serum levels of the reproductive steroid hormones during the menstrual cycle.

Marugo *et al* (1989) note higher receptor concentrations during the proliferative phase, attributing these to the higher levels of circulating oestrogen. They suggest that as a result, tissue levels of the hormone increase, with a subsequent rise in the cytoplasmic production of receptors. This is followed by an increase in the transfer of the hormone receptor complex into the nucleus. During secretory phase, endogenous progesterone blocks the nuclear uptake of the oestrogen hormone complex and hence the receptor levels decline. The problem with this theory is that the receptor has been shown to be a purely nuclear resident and not cytoplasmic as previously thought (Welshons *et al* 1984). Reductions in the quantity and staining of the receptor during the secretory phase have also been observed (Kawaguchi *et al* 1991, Okulicz *et al* 1990, Snijders *et al* 1992).

In the both the present study and that conducted by Chrapusta *et al* (1990) there is no evidence of a menstrual cycle effect in relation to receptor content. The myriad of variation in results is most likely attributable to varying methodologies. In the case of studies which suggest variation with cycle phase, measurements, regardless of technique, have probably isolated the available receptors. In this study the total receptor content has been assessed as opposed to available sites. This was achieved by altering the technique for antigen retrieval by microwaving the tissue prior to the antibody applications. If total receptor content is a constant factor then there should be no variation in the values obtained during the cycle. This in turn would make total content measurements invaluable in the assessment of abnormal states.

3.4.4) *Hoffman Modulation Contrast Microscopy*

For the purposes of this study Hoffman Modulation Contrast microscopy was used. This form of microscopy eliminates tissue variations in the intensity of staining in terms of light reflection. It also offers a 3D type picture of the cells and enhancement of the visualisation of DAB. Being an operator independent system it is an accurate form of microscopy for quantification purposes as it allows for repeatability. A number of scoring systems are available that take into account the intensity of staining (McCarty *et al* 1985), however many of these systems, although accurate and repeatable are cumbersome and time consuming. In this study the form of microscopy used has eliminated certain variable parameters making the scoring of nuclear staining more simplistic and easier to interpret. In general the differences in the percentage nuclear staining seen using this method reflect the pattern of oestrogen positivity within the myometrium. Where, the subendometrial

region not only has a higher percentage of positive cells, but the cells also show increased amounts of reaction product when compared to those of the subserosal region.

3.5) *Conclusion for Immunocytochemical Study One*

Normal myometrium is not uniform, throughout its depth, for the distribution of the oestrogen receptor. Variations in the receptor content between the fundal and lower segment regions of the uterus are not demonstrated.

In spite of the changes that occur in the serum levels of the reproductive steroid hormones, during the menstrual cycle, the myometrial oestrogen receptor levels remain unaffected. Finally this study confirms the importance of standard sampling protocols when assessing myometrial oestrogen receptors.

Table 7: Oestrogen Receptor Staining Of Normal Myometrium In Proliferative Phase

Region	Fundal Region						Lower Segment					
Area	Subendometrial		Midmyometrial		Subserosal		Subendometrial		Midmyometrial		Subserosal	
	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=15	Pos/HPF n=15	Tot cells/HPF n=15	Pos/HPF n=15	Tot cells/HPF n=15	Pos/HPF n=15
	133	109	66	51	54	33	120	97	58	49	30	22
	116	83	80	67	34	31	100	84	47	32	37	27
	112	86	86	61	46	15	118	91	40	30	39	17
	115	99	91	65	30	21	136	126	52	35	41	18
	98	71	66	53	33	9	120	94	57	43	46	26
	122	110	55	43	25	19	101	70	63	36	31	20
	109	94	75	62	38	21	74	76	42	37	34	7
	122	109	52	39	11	7	71	66	79	20	41	12
	104	82	47	36	44	26	89	76	60	29	47	13
	87	75	69	43	26	15	106	126	61	42	42	10
	111	89	50	26	40	15	119	125	55	21	42	37
	118	91	52	30	36	16	107	96	66	30	43	25
	102	86	46	39	30	14	97	77	36	35	27	14
	83	66	46	20	35	6	81	62	45	17	32	13
	125	76	50	25	33	7	140	136	66	30	31	19
	112	80	54	24	55	16						
	117	87	59	29	50	10						
	126	97	60	27	30	17						
	115	85	57	26	46	14						
	105	86	50	24	35	16						
Mean	111.70	88.15	60.55	39.70	36.75	16.70	105.27	94.13	55.53	32.40	37.53	18.67
Max	133	110	91	67	55	33	140	136	79	49	47	37
Min	83	66	46	20	11	7	71	62	36	17	27	7
Std Dev	12.66	12.06	13.51	15.25	10.61	7.36	20.60	24.37	11.66	8.77	6.29	7.83
% Pos		78.92		65.57		45.44		89.42		58.34		49.73

Table 8: Oestrogen Receptor Staining Of Normal Myometrium In Secretory Phase

Region	Fundal Region						Lower Segment					
Area	Subendometrial		Midmyometrial		Subserosal		Subendometrial		Midmyometrial		Subserosal	
	Tot cells/HPF n=30	Pos/HPF n=30	Tot cells/HPF n=30	Pos/HPF n=30	Tot cells/HPF n=30	Pos/HPF n=30	Tot cells/HPF n=25	Pos/HPF n=25	Tot cells/HPF n=25	Pos/HPF n=25	Tot cells/HPF n=25	Pos/HPF n=25
	118	98	49	18	39	14	84	75	38	26	31	23
	118	108	80	27	46	14	82	78	44	33	40	20
	117	103	88	63	39	14	93	83	68	48	26	8
	113	88	48	9	42	19	87	75	79	55	35	23
	75	57	64	31	45	12	78	73	47	35	39	11
	92	82	55	45	39	22	113	92	49	35	35	26
	98	88	68	54	32	15	125	101	66	52	44	22
	130	115	74	59	36	12	156	119	88	58	39	32
	68	62	77	59	44	27	113	92	60	46	52	39
	134	112	41	23	43	27	82	68	58	37	26	6
	159	131	61	48	42	36	80	72	39	29	27	18
	159	132	77	64	43	25	90	82	46	35	33	13
	112	93	67	50	53	36	74	60	77	64	21	11
	101	74	86	75	49	40	100	93	60	45	23	14
	85	69	54	31	60	32	88	77	88	69	31	19
	80	72	39	29	27	18	94	84	43	29	26	13
	90	82	46	35	33	13	67	59	62	38	51	29
	74	60	77	64	21	11	107	98	62	52	46	24
	100	93	60	45	23	14	100	95	47	22	32	11
	88	77	88	69	31	19	84	65	64	57	57	23
	94	89	40	24	28	22	155	121	99	69	53	18
	114	104	39	26	32	23	129	118	77	37	47	13
	103	94	64	39	32	22	130	116	92	23	44	12
	122	102	74	64	34	15	125	102	92	55	56	20
	109	101	58	45	31	15	113	94	94	39	34	18
	147	118	75	41	65	28						
	110	84	100	61	53	19						
	110	79	66	45	50	29						
	127	103	79	53	72	22						
	120	102	63	47	30	8						
Mean	108.9	92.4	65.23	44.77	40.47	20.77	101.96	87.68	65.56	43.52	38.16	18.72
Max	159	132	100	75	72	40	156	121	99	69	57	39
Min	68	57	39	9	21	8	67	59	38	22	21	6
Std Dev	23.12	19.29	16.22	16.76	12.01	8.22	24.08	18.27	19.05	13.88	10.46	7.90
% Pos		84.85		68.63		51.32		85.99		66.38		49.06

Table 9: Percentage Nuclear Staining Of Normal Myometrium In Proliferative Phase

Area	Subendometrial n=12			Midmyometrial n=12			Subserosal n=12		
% Stain	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%
	13	46	41	16	66	18	49	47	4
	7	23	70	38	49	13	62	34	4
	8	47	45	17	63	20	56	26	18
	31	25	44	41	52	7	86	12	2
	8	23	69	17	63	20	57	36	7
	5	36	59	17	56	27	58	31	11
	3	31	66	26	66	8	65	30	5
	11	31	58	11	70	19	85	14	1
	5	32	63	14	74	12	65	34	1
	5	19	76	8	60	32	63	31	6
	4	11	85	14	56	30	63	27	10
	7	24	69	20	71	9	62	35	3
Mean	8.92	29.00	62.08	19.92	62.17	17.92	64.25	29.75	6.00
Max	31	47	85	41	74	32	86	47	18
Min	3	11	41	8	49	7	49	12	1
Std Dev	7.53	10.51	13.43	10.18	7.79	8.49	10.91	9.48	4.95
% Area	8.92	29.00	62.08	19.92	62.17	17.92	64.25	29.75	6.00

Table 10: Percentage Nuclear Staining Of Normal Myometrium In Secretory Phase

Area	Subendometrial n=11			Midmyometrial n=11			Subserosal n=11		
% Stain	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%
	3	32	65	24	67	9	70	27	3
	1	36	63	15	64	21	27	62	11
	9	30	61	21	64	15	34	60	6
	4	25	71	17	66	17	62	31	7
	5	17	78	30	61	9	66	32	2
	6	16	78	41	49	10	75	23	2
	5	18	77	18	55	27	77	22	1
	14	34	52	35	53	12	73	20	7
	7	47	46	44	44	12	82	15	3
	14	75	11	78	21	1	92	7	1
	8	17	75	29	54	17	77	18	5
Mean	6.91	31.55	61.55	32.00	54.36	13.64	66.82	28.82	4.36
Max	14	75	78	78	67	27	92	62	11
Min	1	16	11	15	21	1	27	7	1
Std Dev	4.16	17.45	19.88	18.05	13.33	6.92	19.68	17.42	3.14
% Area	6.91	31.55	61.55	32.00	54.36	13.64	66.82	28.82	4.36

4) IMMUNOCYTOCHEMICAL STUDY TWO

4.1) *Study Aim*

This study is aimed at establishing firstly, whether there is a differential distribution of oestrogen receptors in leiomyomatous myometria and comparing the values obtained to those of normal myometrium. Secondly, whether normal and leiomyomatous myometria respond in a like manner, in terms of oestrogen receptor content, to the variations in serum reproductive steroid hormones in the normal endometrial cycle.

4.2) *Materials and Methods*

3.2.1) *Patient Sample*

A total of thirty uteri from patients in the 31 to 50 year old age group, with no history of exogenous reproductive steroid hormone intake, were selected for this study. Of the selected uteri ten were in the proliferative phase of the endometrial cycle while the remaining twenty were in the secretory phase. Five blocks of fundal tissue from the secretory phase group were unsuitable for use as their endometrium had been resected.

4.2.2) *Methods*

The tissue for this study was collected and stored as detailed in chapter five. Both the processing and immunocytochemical staining were carried out in precisely the same manner as for the normal myometria studied in immunocytochemical study one, with the exception that radioimmunoassay of the samples was excluded.

Endometrial cycle phase was confirmed by brightfield microscopy of the haematoxylin and eosin stained sections. Immunocytochemically stained sections were viewed by Hoffman Modulation Contrast microscopy, with cell counting carried out as for immunocytochemical study one, where both the fundus and lower segment were divided into subendometrial, midmyometrial and subserosal areas respectively. Total cell populations, positive stained cells and percentage nuclear staining were assessed for each area from the fundal and lower segment regions.

4.3) *Results*

As in immunocytochemical study one brightfield microscopy confirmed that the dissected blocks of leiomyomatous myometrium had been removed from the correct areas of the uteri and that the tissue was in either the proliferative or secretory phase.

Myometrium of leiomyomatous uteri responds to immunocytochemical staining for the oestrogen receptor in much the same way as normal myometrium, where the DAB reaction product is isolated to the nucleus of positive cells and does not stain up any of the blood vessel nuclei.

As can be seen from Tables 11 and 12 the subendometrial area is not only the most cellular area, with an average of 100 nuclei per high power field, but it also predominates in respect of oestrogen positivity where over 86% of all nuclei counted are oestrogen receptor positive. The midmyometrium is approximately half as cellular as the subendometrium with only 54 nuclei per high power field. However, nuclear positivity remains high in this region with more than 77% of the total nuclei demonstrating DAB reaction product. With only 34 nuclei per high power field the subserosa is the least populated of the three areas. Positivity in this outlying area is only marginally less than that of the midmyometrium and averages 72% of counted nuclei.

Analysis of variance testing conducted in conjunction with the normal myometrial results, from immunocytochemical study one, fails to show any significant difference in positivity between the fundus and lower segment regions ($p < 0,6549$). Variation in the counts for positivity of the three areas, of leiomyomatous myometria, may appear to be small but are in fact significant ($p < 0,001$). Similar to normal myometrium the phase of the endometrial cycle does not significantly affect the immunopositivity of either the regions or areas of the leiomyomatous myometrium ($p < 0,5593$).

Comparisons of the data from normal and leiomyomatous myometria can be made as the cell counting for both was conducted by a single operator, with intermingling of the samples, so as to ensure that all samples were treated in the same manner. Little difference exists between normal and leiomyomatous myometria with regard to the total cellularity for each respective area counted. The

percentage of the nucleus obscured by reaction product is similar for both normal and leiomyomatous myometria (Table 13). For both groups of tissue the nuclei of the subendometrial areas are more heavily stained than the middle and outer areas.

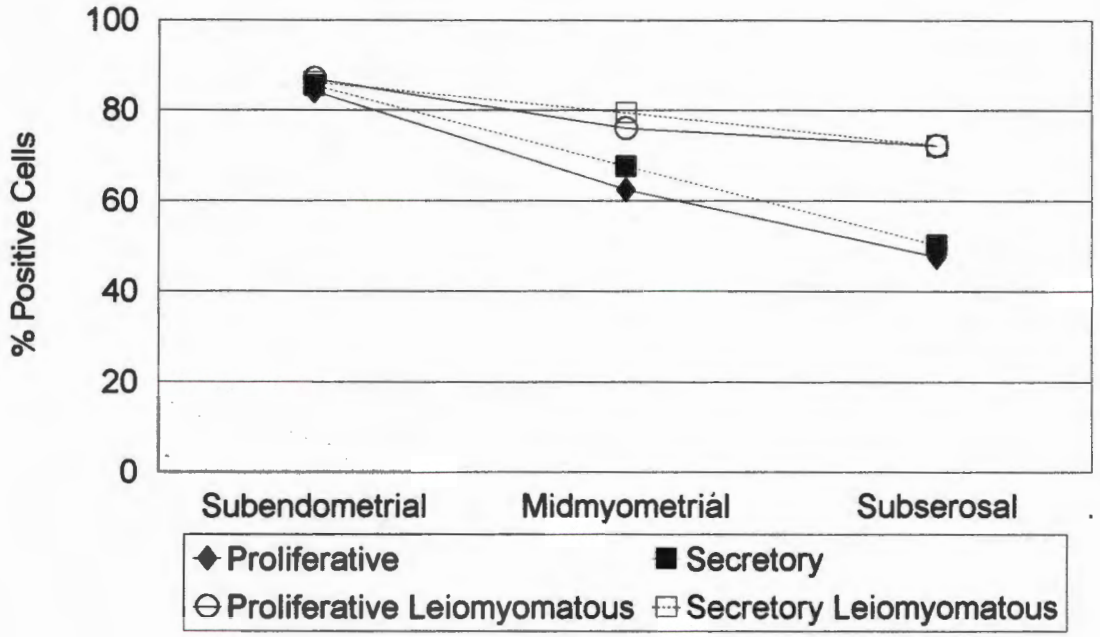

Figure 38: Graphical representation of the oestrogen receptor content of normal and leiomyomatous myometria during the proliferative and secretory phases of the endometrial cycle.

Figure 38 demonstrates the differences in the positivity between normal and leiomyomatous myometria in terms of area and endometrial cycle phase. As already stated the phase of the endometrial cycle does not significantly interact with the degree of positivity. There is however a significant difference in the positivity between normal and leiomyomatous myometria ($p < 0,0001$), where the myometrium of tumorous uteri have a significantly greater population of oestrogen positive nuclei.

4.4) *Discussion*

4.4.1) *The Importance of Sampling*

Researchers of the oestrogen receptor content of leiomyomata have in general assumed that the host myometrium is inherently normal. They have therefore tended to use the leiomyomatous myometrium for control sampling (Chrapusta *et al* 1990, Farber *et al* 1972, Kawaguchi *et al* 1991, Marugo *et al* 1989, Otsuka *et al* 1989, Pollow *et al* 1978, Soules & McCarty 1982, Wilson *et al* 1980). However, there appear to be no studies which specifically compare the oestrogen receptor content and distribution in leiomyomatous myometrium to that of normal myometrium. The results of this study indicate that the distribution and content of oestrogen receptors in the myometria of normal and leiomyomatous uteri differ significantly from each other. Thus in studying leiomyomata control sampling must be made from a separate population of normal uteri.

4.4.2) *Response to the Endometrial Cycle*

Neither normal nor leiomyomatous myometrium demonstrate alterations in their oestrogen receptor status during the menstrual cycle. This is probably attributable to the total receptor content of these tissues remaining constant during the cycle. In other words an equivalent number of receptors are produced as are utilised. The total number of receptors does however differ between the two varieties of tissue. As both respond to the normal variations in the serum levels of the reproductive steroid hormones in an apparently similar fashion, the response of leiomyomatous myometrium can be considered to be essentially normal.

4.4.3) *Transmural Oestrogen Receptor Gradient*

The transmural oestrogen receptor concentration gradient is markedly flattened in comparison to that of normal myometrium. Both the midmyometrial and subserosal areas of leiomyomatous myometrium are far more heavily populated with immunopositive cells than their normal counterparts. As a result the overall oestrogen receptor content of the leiomyomatous myometrium is increased.

It is not possible, from these samples, to assess whether the presence of leiomyomata is responsible for the increase in the oestrogen receptor content of the

surrounding myometrium or not. Considering that leiomyomata are rarely solitary and that they tend to recur, subsequent to myomectomy, suggests a primary abnormality in the host myometrium. The increased amount of oestrogen receptors within the myometrium may produce a state of heightened irritability and responsiveness, sufficient to act as a major predisposing factor for leiomyomata formation.

The fact that most leiomyomata arise in the midmyometrial and subserosal areas of affected uteri, while the minority occur in the subendometrium, lends support to the increased receptor concentration acting as a predisposing factor. This can be explained in terms of the concentration ratio for normal to leiomyomatous uteri in each of the three transmural areas. Within the subendometrium an almost 1:1 ratio for positivity exists between normal and abnormal myometria and thus the difference is insufficient to act as a major factor in tumour formation. In the remaining two thirds of the myometrial wall the ratio changes to 1:1.3 and 1:1.5 for the middle and outer areas respectively. Thus there is a 30% to 50% increase in the positivity of these regions in comparison to the normal. Such an increase may well aid in providing the necessary nidus for tumour formation.

Ideally the theory should be tested by examining a young population of normal uteri to determine whether the abnormality is present and then to follow up such cases to assess whether or not they produce leiomyomata. Unfortunately an *in vivo* study of this nature is not possible within the human population and animal models are not suitable due to the human specific nature of this phenomenon.

4.5) *Conclusion for Immunocytochemical Study Two*

Even though leiomyomatous myometrium demonstrates a similar differential pattern of oestrogen receptor distribution, the distribution curve is flattened in comparison to its normal counterparts. Like normal myometrium, the oestrogen receptor content of leiomyomatous myometrium does not differ from the fundus to the lower segment. The myometrium of a leiomyomatous uterus may be considered to be abnormal, by virtue of its higher levels of oestrogen receptor positivity, when compared to that of a normal uterus. Despite its high degree of positivity, leiomyomatous myometrium responds in the same manner as normal myometrium, to the cyclical variations in the serum levels of the reproductive steroid hormones.

Table 11: Oestrogen Receptor Staining Of Leiomyomatous Myometrium In Proliferative Phase

Region	Fundal Region						Lower Segment					
Area	Subendometrial		Midmyometrial		Subserosal		Subendometrial		Midmyometrial		Subserosal	
	Tot cells/HPF n=10	Pos/HPF n=10	Tot cells/HPF n=10	Pos/HPF n=10	Tot cells/HPF n=5	Pos/HPF n=5	Tot cells/HPF n=10	Pos/HPF n=10	Tot cells/HPF n=10	Pos/HPF n=10	Tot cells/HPF n=10	Pos/HPF n=10
	98	93	75	54	25	17	121	105	45	10	40	27
	114	90	33	30	11	9	112	95	70	59	23	19
	108	94	53	48	33	31	113	99	82	75	35	27
	92	80	45	34	40	29	99	89	72	54	52	43
	80	73	56	54	24	11	92	81	85	74	28	18
	102	96	54	44			141	128	81	62	61	43
	115	98	33	28			123	105	63	44	50	32
	124	92	39	20			101	94	66	51	82	54
	102	88	52	25			71	62	81	56	58	41
	87	68	54	42			116	107	70	54	51	38
Mean	102.20	87.2	49.40	37.90	26.60	19.40	108.90	96.50	71.50	53.90	48.00	34.20
Max	124	98	75	54	40	31	141	128	85	75	82	54
Min	80	68	33	20	11	9	71	62	45	10	23	18
Std Dev	13.57	10.15	12.59	12.19	10.88	10.14	19.25	17.48	11.94	18.19	17.36	11.59
% Pos		85.32		76.72		72.93		88.61		75.38		71.25

Table 12: Oestrogen Receptor Staining Of Leiomyomatous Myometrium In Secretory Phase

Region	Fundal Region						Lower Segment					
Area	Subendometrial		Midmyometrial		Subserosal		Subendometrial		Midmyometrial		Subserosal	
	Tot cells/HPF n=15	Pos/HPF n=15	Tot cells/HPF n=15	Pos/HPF n=15	Tot cells/HPF n=15	Pos/HPF n=15	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=20	Pos/HPF n=20
	119	101	39	28	46	18	71	62	49	45	33	28
	87	78	73	55	29	16	100	89	61	58	27	21
	100	87	54	46	38	33	94	85	41	25	25	18
	108	97	48	43	21	17	134	117	45	31	21	18
	113	103	53	41	49	36	98	91	44	33	4	4
	77	66	53	47	22	17	78	76	39	27	32	24
	100	92	69	58	30	21	84	81	62	48	24	15
	74	67	44	40	34	27	90	76	58	44	36	26
	100	81	43	35	30	22	80	67	47	36	45	31
	88	77	48	49	32	19	102	91	48	38	51	41
	113	79	36	25	35	28	90	79	26	2	17	14
	111	95	52	42	40	31	96	86	28	24	24	20
	74	57	74	57	25	18	100	88	44	39	18	13
	92	84	55	47	22	14	87	73	42	31	27	22
	104	78	45	31	27	20	79	53	28	22	23	16
							76	66	55	51	24	17
							68	60	53	38	26	19
							113	100	37	27	36	26
							74	62	55	43	29	9
							103	84	47	38	32	28
Mean	97.33	82.80	52.40	42.93	32.00	22.47	90.85	79.30	45.45	35.00	27.70	20.50
Max	119	103	74	58	49	36	134	117	62	58	51	41
Min	74	57	36	25	21	14	68	53	26	2	4	4
Std Dev	14.76	13.33	11.58	10.05	8.49	6.82	15.87	15.30	10.45	12.32	10.09	8.27
% Pos		85.07		81.93		70.21		87.29		77.01		74.01

Table 13: Percentage Nuclear Staining Of Leiomyomatous Myometrium In Proliferative/Secretory Phase

Area	Subendometrial n=10			Midmyometrial n=10			Subserosal n=10		
% Stain	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%
	7	10	83	27	52	21	58	31	11
	15	56	29	28	50	22	65	31	4
	3	15	82	20	69	11	64	30	6
	9	14	77	32	54	14	56	40	4
	11	11	78	12	58	30	25	39	36
	19	55	26	34	43	22	56	33	11
	3	12	85	12	60	28	61	28	11
	19	53	28	48	47	5	65	32	3
	17	40	43	19	50	31	51	34	15
	11	22	67	18	51	31	35	39	26
Mean	11.40	28.80	59.80	25.00	53.40	21.50	53.60	33.70	12.70
Max	19	56	85	48	69	31	65	40	36
Min	3	10	26	12	43	5	25	28	3
Std Dev	6.02	19.85	25.23	11.16	7.37	9.03	13.43	4.22	10.67
% Area	11.40	28.80	59.8	25.00	53.4	21.5	53.6	33.7	12.70

5) IMMUNOCYTOCHEMICAL STUDY THREE

5.1) *Study Aim*

The primary aim of this investigation is to establish whether leiomyomata and normal myometrium differ with respect to their oestrogen receptor content. Secondly, the oestrogen receptor content of both large and small leiomyomata are compared and thirdly the response of these tumours to the normal hormonal cyclical variations of the menstrual cycle is examined.

5.2) *Materials and Methods*

5.2.1) *Patient Sample*

Leiomyomata from a total of fifty five patients, between the ages of 31 and 50 years, with no history of exogenous reproductive steroid hormone intake, were selected for this study. Thirty of the selected uteri were from patients in the proliferative phase of the endometrial cycle as at the time of surgery. From these thirty uteri a total of thirty small and twenty large leiomyomata were suitable for the purposes of the study. The remaining twenty five uteri, all in the secretory phase, yielded 25 small and 15 large tumours for examination.

5.2.2) *Methods*

Selected leiomyomata were designated as small if their diameter measured less than 3cm and large if it was greater than 3cm. The blocks of tissue excised from the tumours were fixed and processed to wax as detailed in chapter five. Oestrogen receptor localisation by immunocytochemistry was performed in exactly the same manner as for the tissue in immunocytochemical studies two and three.

Brightfield microscopy was used to confirm that the leiomyomata selected for the study were of the common type. As in the previous two studies the immunostained sections were viewed by Hoffman Modulation Contrast microscopy. The counting procedure differed slightly to the previous studies as the sections were not divisible, into specific areas, in the same manner as were the myometrial samples. The cell populations of ten randomly selected areas per slide were counted

where both the total number of cells per high power field, as well as the total number of positive nuclei per high power field, were recorded.

In a random selection of samples the percentage nuclear staining was assessed according to the same criteria as for the nuclei in the first two immunocytochemical studies. Nuclei were recorded as either less than one third stained, between one and two thirds stained or as greater than two thirds stained.

5.3) *Results*

All leiomyomata used in this study were confirmed to be of the common type following Brightfield examination of the haematoxylin and eosin sections.

The DAB reaction product is limited to the nuclei of positive stained cells and is not found in the interstitium or in the blood vessels. The values obtained for both the total and positive cells counted per high power field, in both small and large leiomyomata are presented in relation to the phase of the endometrial cycle in Table 14 (Figure 39). The characteristics of nuclear staining for both small and large leiomyomata are detailed in Table 15.

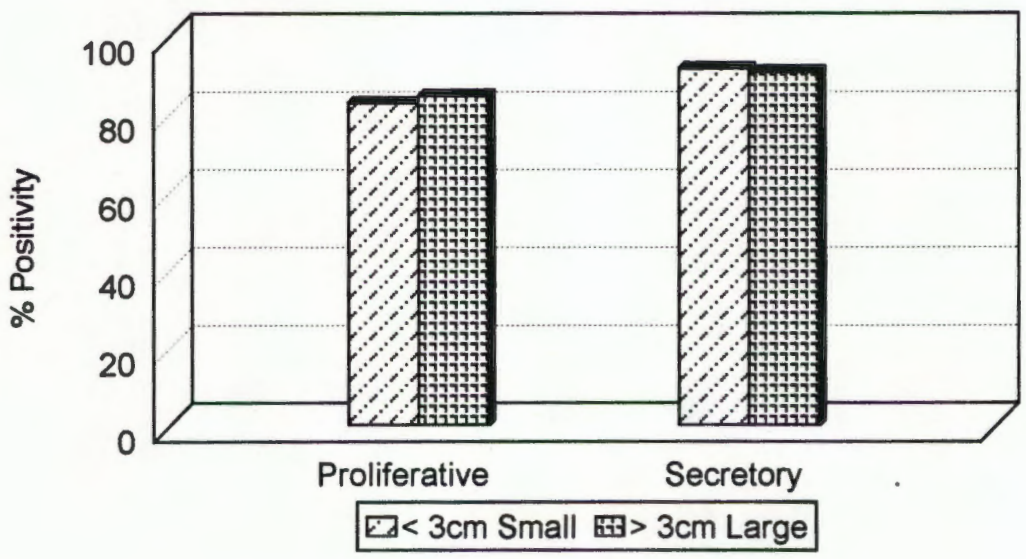

Figure 39: Graphical representation of the oestrogen receptor content of large and small leiomyomata during the proliferative and secretory phases of the endometrial cycle.

Although the mean values for the total number of cells per high power field does not vary greatly between the small and large tumours, there is a marked degree of variation between the individual tumours within each group. Some sections counted display as few as 8 cells per high power field, where others are similar to the subendometrial region of the myometrium, with cellularities of over 100 cells per high power field.

Leiomyomata, regardless of their size are highly positive for the oestrogen receptor. Over 80% of nuclei of proliferative phase and 90% of secretory phase tumours respectively are postive for the oestrogen receptor. The Mann-Whitney test, a non-parametric equivalent of the t-test does not reveal any significant difference between the positivity of small and large tumours of the same endometrial cycle phase. A significant difference does exist between the small proliferative phase and small secretory phase leiomyomata ($p < 0,0037$). Likewise, large proliferative phase and large secretory phase leiomyomata are significantly different ($p < 0,027$) with respect to positivity.

The percentage of the nucleus obscured by reaction product is a highly variable phenomenon in respect of leiomyomata. In small leiomyomata only 10% of nuclei are less than one third stained while in large leiomyomata there is an even distribution of staining of nuclear staining (Table 15).

5.4) *Discussion*

The issue of whether or not leiomyomata differ from normal myometrium with regards their receptor content has in the past been extensively debated. Results of research range greatly with some suggesting that leiomyomata are richer than the myometrium for the oestrogen receptor (Farber *et al* 1972, Kawaguchi *et al* 1991, Otsuka *et al* 189, Pollow *et al* 1978), while others can find no such difference (Marugo *et al* 1989, Wilson *et al* 1980). The major problem with the majority of these studies is that the assessments have been made on the results of radioimmunoassay techniques. Thus any minor differences in sample preparation and technique, between the laboratories, will be magnified in the results obtained. This makes comparing the results impossible.

The results of the present study indicate that leiomyomata are in fact significantly richer in oestrogen receptors than the midmyometrial and subserosal

regions of the normal myometrium but do not differ greatly to normal subendometrial myometrium. The assessments of Marugo *et al* (1989) and Soules and McCarty (1982), that leiomyomata may be marginally richer in receptors than their host myometrium, but not significantly so, is by serendipity in agreement with the present study. In both cases they have assumed that the host myometrium is normal in respect of its receptor content while present research has shown it is not. In fact the average oestrogen receptor content of both large and small leiomyomata is only marginally increased in comparison to its surroundings and markedly increased when compared to the average of the three areas of a normal myometrium.

It would appear possible, that if the primary prediposing factor for the formation of leiomyomata exists in a myometrium which is inherently abnormal and that the structure of the resulting tumours does not differ greatly from the surroundings, then nor will their receptor status. This is indeed the case for the leiomyomata examined in this study.

According to a qualitative study by Kawaguchi *et al* (1991) immunocytochemical staining for the oestrogen receptor persists throughout the menstrual cycle with no evidence of fluctuation. Quantitative evaluation of staining for both small and large leiomyomata shows that the receptor content for both is significantly increased during the secretory phase of the menstrual cycle, even though the magnitude of the increase appears to be small. The differences between the phases of the cycle may be a reflection of the ablilty of the tumours to alter their local hormonal environment. It has been suggested that these tumours are capable of synthesizing oestrogens by aromatisation of circulating androgens with a resultant increase in receptor production (Chrapusta *et al* 1990).

Regardless of the cause of the increase in the receptor content of these tumours they are by no means uniform with regard to their distribution of the oestrogen receptor. Some tumour areas demonstrate almost no positivity whilst others are almost 100% positive. The percentage of the nucleus obscured by reaction product is also highly variable and no distinct pattern is seen. Small leiomyomata do however tend to have very few nuclei with less than 33% of the nucleus obscured. This may well be an indication of the developing tumours high state of reactivity in terms of oestrogen response.

5.5) *Conclusion for Immunocytochemical Study Three*

Leiomyomata of proliferative phase myometria have an equivalent percentage of immunopositive cells as the subendometrial area of both normal and leiomyomatous myometrium, whereas, secretory phase leiomyomata are richer in oestrogen receptors than both their host and normal myometrium.

Although small and large leiomyomata do not differ from each other with regards their oestrogen receptor content, they respond differently to the phase of the menstrual cycle. Thus leiomyomata and normal myometrium do not respond in a similar fashion to the external stimulus of the menstrual cycle.

Table 14: Oestrogen Receptor Staining Of Leiomyomata

Phase	Proliferative				Secretory			
Size	<3cm (Small)		>3 cm (Large)		<3cm (Small)		>3 cm (Large)	
	Tot cells/HPF n=30	Pos/HPF n=30	Tot cells/HPF n=20	Pos/HPF n=20	Tot cells/HPF n=25	Pos/HPF n=25	Tot cells/HPF n=15	Pos/HPF n=15
	44	42	34	31	33	30	32	32
	35	32	26	25	59	56	64	57
	45	44	63	57	48	47	16	16
	52	52	61	53	48	48	46	42
	64	58	42	38	53	52	34	18
	55	52	42	42	45	42	62	58
	61	52	83	71	45	40	71	68
	46	39	69	63	58	47	44	41
	44	39	62	45	91	81	68	60
	38	3	55	46	67	59	30	29
	37	33	74	54	88	81	24	24
	45	29	52	34	104	94	34	31
	41	35	43	35	65	63	20	18
	44	40	19	16	71	58	41	39
	57	53	74	65	52	50	36	31
	48	45	108	86	69	66		
	31	27	8	7	37	28		
	45	38	28	27	40	36		
	39	35	29	28	55	51		
	40	35	42	34	49	40		
	61	56			33	30		
	67	55			40	39		
	58	43			41	40		
	67	48			48	45		
	59	45			42	41		
	60	48						
	70	59						
	72	60						
	57	42						
	55	33						
Mean	51.23	42.4	50.70	42.85	55.24	50.56	41.47	37.60
Max	72	60	108	86	104	94	71	68
Min	31	3	8	7	33	28	16	16
Std Dev	11.23	11.90	24.22	19.43	18.27	16.53	17.55	16.61
% Pos		82.76		84.52		91.53		90.68

Table 15: Percentage Nuclear Staining Of Leiomyomata

Tumour	Small (<3cm) n=11			Large (>3cm) n=9		
% Stain	less than 33%	33% to 66%	more than 66%	less than 33%	33% to 66%	more than 66%
	3	62	35	8	13	79
	11	15	74	15	17	68
	12	77	11	70	22	8
	15	70	15	34	53	13
	7	15	78	51	34	15
	10	15	75	19	67	14
	13	58	29	46	50	4
	5	18	77	12	31	57
	7	20	73	38	50	12
	16	78	6			
	10	81	9			
Mean	9.91	46.27	43.82	32.56	37.44	30.00
Max	16	81	78	70	67	79
Min	3	15	6	0	13	0
Std Dev	4.09	29.20	31.39	22.82	22.43	28.81
% Area	9.91	46.27	43.82	32.67	37.44	30.00

CHAPTER NINE

CONCLUSION

The underlying purpose of this thesis was to gain an understanding of the fundamental character of leiomyomata and the myometrium in which they occur. To this end three basic questions were asked:

1 Is the non-neoplastic myometrium of leiomyomatous uteri normal or abnormal?

2) What differences in structure and oestrogen receptor content are there between normal myometrium and leiomyomata?

3) Do leiomyomata and myometrium respond in a like manner to changes in age, parity and endogenous oestrogen?

1) STUDY DESIGN

In order to effectively compare leiomyomata to normal myometria it was necessary to define the parameters of normality and then limit, as far as possible, the variable factors. To this end serum analyses for the circulating levels of oestradiol, progesterone and sex hormone binding globulin were conducted for both normal and leiomyomatous patients.

The circulating levels of the hormones were similar in both groups of patients with normal endometria. Significantly higher levels of progesterone occurred in leiomyomatous patients with inactive endometria. The higher progesterone values and consequent altered endometria were attributable to exogenous progestagen therapy prior to surgery. As hormone therapy produced both an altered serum level and structural change it was decided to exclude from the study all patients with a history of exogenous reproductive steroid hormone ingestion.

2) QUESTION ONE

The myometrium that acts as a host for leiomyomata is fundamentally abnormal. At the light microscope level, normal and leiomyomatous myometria are indistinguishable from each other whereas ultrastructurally the major feature of difference is the increase in plasmalemmal densities that occur in leiomyomatous myocytes. The significance of the finding is related to the concomitant decrease in the number of plasmalemmal vesicles in these cells. This decrease may well affect the action potential capacity of the myocyte and thus have a detrimental effect on the contracting ability of the affected myometrium.

As stated in chapter eight it has been generally assumed that the myometrium of leiomyomatous uteri is normal with regard to its oestrogen receptor content. It has thus been commonly used as the control for the study of oestrogen receptors in leiomyomata.

The distribution of oestrogen receptors in normal myometrium was demonstrated to be non-uniform through the depth of the myometrial wall. Where, the subendometrial region was significantly richer in receptors than the midmyometrial and subserosal regions. No difference in oestrogen receptor distribution was noted between the fundal and lower segments of the uterus. When leiomyomatous myometria were compared to normal myometria significant differences in their total receptor content and distribution were discovered. The leiomyomatous myometria were richer than their normal counterparts for oestrogen receptors and although they demonstrated a similar pattern of distribution, the distribution gradient was flattened. Whether the increase in oestrogen receptors is the consequence or cause of leiomyomata is still to be elicited but it appears as if the presence of an abnormal myometrium may well present an ideal nidus for tumour formation. The fact that leiomyomatous myometria cannot be classed as normal means that they cannot be used for control sampling and that where they have been, reassessment is essential.

3) QUESTION TWO

Ultrastructurally, leiomyomata and normal myometrium are two distinct entities. Perhaps the most significant finding was the identification, within

leiomyomata, of a distinct population of myofibrocytes. The presence of these cells supports the unicellular theory of origin and the pleuripotentiality of the myocyte. Like their host tissue, the tumours had increased amounts of plasmalemmal densities and few vesicles.

With regards their oestrogen receptor content, normal myometria and leiomyomata differ significantly. The tumours have a higher percentage of postivity than the mean for that of normal myometrium but are comparable to the levels found within the normal subendometrial region. Tumour oestrogen receptor positivity does not vary much from that of the myometrium in which it is found. The predominance of intramural fibroids is explained in terms of the receptor ratio of normal to abnormal myometrium where the greater the ratio the greater the probability of developing leiomyomata.

4) QUESTION THREE

Leiomyomata and normal myometrium respond ultrastructurally in a similar manner to changes in endogenous oestrogen, in that neither undergoes significant structural alterations during the normal menstrual cycle. They do however differ with regard their oestrogen receptor content during the menstrual cycle. No variation occurs in normal myometrium but marked increases in oestrogen receptor content occur in leiomyomata during the secretory phase. This difference could not be explained from the available data.

The aged normal and leiomyomatous myometrium are similar in that they both display increased amounts of lipofuscin and decreased amounts of lipid-rich residual bodies. Leiomyomata differ in their response to aging in that arise at a later stage and grow at a different rate. The tumours are most difficult to date in relation to their host but it appears as if the larger the tumour the older it is. Aging is thus seen in terms of the increase in the amount of connective tissue that is produced by the tumour in relation to its muscle fibres.

The effects of parity could not be satisfactorily assessed with the available sample in that the sample could not be delimited in terms of age.

5) WHERE TO FROM HERE?

Even though a number of answers have been provided to the questions asked at the beginning of this thesis, there are still many issues related to the basic character of these tumours that still need to be addressed. Further research needs to be aimed at finally understanding the aetiology of these tumours so that practical, realistic forms of therapy can be offered to affected women.

The results of this thesis indicate that leiomyomata are not caused by an abnormality in the circulatory distribution of the reproductive steroid hormones. There is, however, an indication of a primary abnormality of the myometrium in which they occur, where the oestrogen receptor content of both the tumours and their host tissue is increased. It would appear that subcellular studies with regards the cascade factors that lead to receptor formation require investigation.

An analysis of progesterone receptor staining is also warranted. To this end the procedure for staining still requires much work before it can be used for the form of quantification that was carried out for this thesis. Finally, when the baselines for normality and abnormality have been unequivocally established then further studies on the effects of the reproductive steroid hormones on normal and leiomyomatous tissue can be carried out with confidence.

APPENDIX I

STANDARD METHODS

I) RADIOIMMUNOASSAY FOR OESTRADIOL

1) Allow frozen serum to thaw naturally.
2) Extract oestradiol using di-ethyl ether. Evaporate solvent to dryness under a stream of nitrogen. Reconstitute with buffer (Appendix II:VII).
3) Preincubate samples with anti 17beta-oestradiol-6BSA serum (Biomaker) for 30 minutes at room temperature.
4) Add [2,4,6,7,16,17-3H]-oestradiol tracer and incubate at $4^{O}C$ overnight to allow assay to reach equilibrium.
5) Separate bound and free with dextran coated charcoal (Appendix II:VIII).
6) Centrifuge supernatant and count in a liquid scintillation counter.

II) COAT-A-COUNT^{R} PROGESTERONE

The following method is a summary of that supplied with the kit. This procedure is a solid-phase radioimmunoassay where the antibody is immobilised to the wall of a polypropylene tube.

1) Bring all constituents to room temperature.
2) Label four uncoated tubes for the total counts.
3) Label the fourteen calibrator progesterone Ab-coated tubes. Label additional tubes for the patient samples.
4) Pipet 100μl of the different calibrators into the appropriately labelled tubes.
5) Pipette 100μl of patient samples into each relevant tube.
6) Add 1ml of ^{125}I progesterone to each tube and vortex.
7) Incubate for 3 hours at room temperature.
8) Decant thoroughly.
9) Count for 1 minute in a gamma counter.

III) IMMUNORADIOMETRIC ASSAY FOR SHBG

The following method is a summary of the method supplied with the kit for the immunoradiometric assay for sex hormone binding globulin.

1) Bring all constituents to room temperature.
2) Dilute standards, control sera and samples to 1:100.
3) Vortex mix the dilutions.
4) Label a duplicated series of tubes for the total counts, non-specific binding, the standards, the controls and the samples.
5) Mix together equal quantities of anti-SHBG antiserum and [^{125}I] anti-SHBG antibody.
6) Add 200μl of above mixture to all tubes.
7) Vortex and incubate for 1 hour at room temperature.
8) Add 500μl of stirred separation agent to each tube except totals.
9) Vortex and add 2ml 0,9% saline to all tubes except totals.
10) Centrifuge at room temperature for at least 15 minutes.
11) Decant supernatant to waste.
12) Count residual activity in a gamma scintillation counter.

IV) STANDARD HAEMATOXYLIN AND EOSIN STAIN

1) Dewax sections in xylol.
2) Rehydrate sections through a graded series of alcohols to water.
3) Stain with Mayer's Haematoxylin (Appendix II:XIII) for 5 minutes.
4) 'Blue' in running water for up to 5 minutes.
5) Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5 to 10 seconds.
6) 'Blue' again in running water for up to 5 minutes.
7) Stain in 1% eosin for 10 minutes.
8) Wash in running tap water for 1 to 5 minutes.
9) Dehydrate through the graded alcohols, clear in xylol and mount.

V) PROCESSING FOR ELECTRON MICROSCOPY

1) Place freshly dissected tissue in a Paraformaldehyde and Glutaraldehyde fixative (Appendix II:I) for 1 hour.
2) Wash in three changes of phosphate buffer (Appendix II:II) for 5 minutes each.
3) Leave sample in phosphate buffer overnight.
4) Post-fix using 1% Osmium tetroxide (Appendix II:III) for 30 minutes at room temperature.
5) Wash in three changes of phosphate buffer for 5 minutes each.
6) Dehydrate samples through a graded series of alcohols in ascending order from 50% to 90%, for 10 minutes in each alcohol, at room temperature.
7) Leave in 100% alcohol for two changes of 10 minutes each at room temperature.
8) Mix spurr resin (Appendix II:IV) with 100% alcohol in a 50:50 ratio and leave sample in mixture for 1 hour at room temperature.
9) Leave sample overnight in pure resin without accelerator in an oven at $40^{o}C$.
10) Mix resin with accelerator and leave sample for 1 hour at room temperature.
11) Embed and label samples prior to placing them in an oven at $60^{o}C$, for at least 48 hours, to allow for polymerisation.

VI) URANYL ACETATE AND LEAD CITRATE STAINING

1) Centrifuge a small quantity of saturated uranyl acetate (UA)(Appendix II:V) and pipette off supernatant.
2) Place single drop of UA on a piece of dental wax.
3) Float grid with section face down on the UA for 10 minutes.
4) Rinse grid through three consecutive drops of distilled water for 5 minutes each.
5) While grid is on final drop of water, filter lead citrate (LC)(Appendix II:VI) through a 0,25μm millipore filter. Discard the first drop and place the second drop of LC in a covered petri dish with a few pellets of NaOH.
6) Float the grid section side down on the LC for 10 minutes.
7) Rinse grid through three consecutive drops of distilled water for 5 minutes each.
8) Blot dry and store in a covered container.

VII) SINGLE POINT RADIOIMMUNOASSAY METHOD

Specimen Collection

1) Immerse uterine tissue in ice water ($0^{o}C$) as soon as possible after surgical removal. It is imperative that the tissue remain cooled until it is frozen in liquid nitrogen as steroid receptors are extremely labile. Improper handling results in false receptor negatives.
2) Cut $1cm^3$ blocks from the fundal and lower segments of the cooled or fresh uterus (0,5g max and 0,2g min).
3) Place blocks in appropriately labelled tin foil bags and immerse in liquid nitrogen to freeze.
4) Store frozen tissue at $-70^{o}C$ until required.

Steroid Receptor Assay

Tissue Preparation

1) Weigh tissue on a top loading balance in a dish preset to zero.
2) Place the frozen tissue samples into a pre-cooled hard plastic container and pulverize the frozen tissue, for 30 seconds, into a fine powder using a pre-cooled thermovac auto-pulveriser (Thermovac industries) (or equivilent).
3) Transfer the powdered tissue using a liquid nitrogen cooled metal spatula into a small pre-cooled plastic beaker which is able to withstand low temperatures.
4) Rest the beaker on ice and add a 10 fold (tissue weight in grams / buffer volume in mls) volume of TRIS buffer (Appendix II:VII).
5) Homogenise the tissue suspension with three bursts, of 15 seconds each, using a Poltron PCU-2 Kinematica (or equivalent) at a setting of six. Hold the beaker on a bed of ice while homogenizing.
6) Transfer the homogenate to a polycarbonate ultracentrifuge tube using a cold pasteur pipette. Wash the beaker out with a further 0,5ml of TRIS buffer to retrieve any remaining homogenate.
7) Centrifuge the homogenate at 30 000rpm, for 40 minutes, at $4^{o}C$ using a Beckmann ultracentrifuge. Pre-cool the centrifuge rotor in the fridge. High speed centrifugation is necessary to sediment out components such as

microsomes and lysosomes, that may lead to high non-specific binding in the receptor assay.

8) Remove the polycarbonate tubes from the centrifuge and place then on ice.

9) Transfer the high speed cytosol supernatant to cold glass test tubes (Kimble tubes 12mm x 75mm of disposable borosilicate glass) utilising a pasteur pipette. Store tubes on ice in a fridge at $4^{o}C$.

Dose Preparation

1) Using two 20ml glass Kimble disposable scintillation vials, place 6μl of radio active estradiol (hot) in the first, label it ER^{*} (hot) and allow the alcohol to evaporate. Add 10mls of TRIS buffer to the vial and vortex it for five minutes.

2) Remove 5mls of fluid from the first vial and place it in the second vial, labelled ER^{o} (cold). To this vial add 6μl of the specific binder diethylstilbestrol (DES).

3) Store both the glass vials on ice.

4) Into three plastic scintillation vials, labelled Blank, ER^{*} and ER^{o} respectively, place 5mls of toluene scintillation fluid and 200μl of TRIS buffer.

5) To the plastic vial marked ER^{*}, add 50μl of fluid from the glass bottle of the same label. Then repeat the equivilent procedure for the vials marked ER^{o}. Store the vials on ice.

6) Prepare four Kimble disposable borosilicate glass tubes for each tissue sample, 2 'hot' (radioactive) and 2 'cold'.

7) In the 'cold' tubes place 100μl of cytosol and 100μl of ER^{o}. Perform the equivilant procedure for the hot tubes using ER^{*}.

8) Vortex each tube and then incubate each tube on a bed of ice in a cold room overnight.

Addition of Charcoal

1) Make up a 1:10 dilution of dextran coated charcoal stock (Appendix II:VIII) to distilled water and stir, on ice, for 5 minutes.

2) Vortex each incubation tube prior to the addition of 500μl of dextran coated charcoal.

3) Incubate the tubes for 30 minutes at $4^{o}C$.

4) Centrifuge the tubes at 3000rpm for 10 minutes in order to sediment the charcoal.

Sample Counting

1) Into plastic scintillation vials, labelled the same as the glass incubation tubes, place 5mls of toluene scintillation fluid and 500μl of supernatant.
2) Count the samples for 5 minutes each on a tritium wide window channel.

VIII) PROTEIN ASSAY FOR RIA

Reagents

1) Bovine serum albumin (BSA) diluted by half with TRIS (Appendix II:VII) to a final concentration of 1,75 mg/ml.
2) Phosphoric acid (2mls)(Biorad agent/Substrate) + TRIS (8mls), filtered to avoid crystal precipitation.

Procedure

1) To obtain the standard curve compose 5 Kimball disposable borosilicate glass tubes as follows:

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
%BSA	100	80	60	40	20
TRIS	0μl	20μl	40μl	60μl	80μl
BSA	100μl	80μl	60μl	40μl	20μl
Protein	1,75mg/ml	1,4mg/ml	1,05mg/ml	0,7mg/ml	0,35mg/ml

2) For each of the samples to be measured use 50μl of cytosol added to 100μl of TRIS buffer.
3) Using a microtitre plate, place 5μl of substrate into each well in row 1, in order to obtain a zero reading.
4) Leave row 2 empty.
5) Place 5μl of the standard curve solution, in decreasing order of concentration, into sequential wells in rows 3 and 4.
6) In rows 5 and 6 place 5μl of the sample solutions into the wells.

7) Allow colour to develop for 5 minutes.
8) Measure the optical density (light absorbance) using a spectrometer set at 620nm.

IX) STREPTAVIDIN-BIOTIN-COMPLEX METHOD

Reagents

1) 3% H_2O_2 in distilled H_2O.
2) 5% Normal Rabbit Serum in TRIS buffered saline (Appendix II:IX).
3) Monoclonal antibody: Oestrogen Receptor 1:50
4) Biotinylated-rabbit anti-mouse.
5) DAB medium.

Technique

1) Incubate 1μm wax sections on glass slides at 60^{o}C for 20 to 30 minutes in order to prevent sections from floating off during subsequent procedures.
2) Dewax sections in xylol for 5 minutes.
3) Hydrate sections by passing them through a graded alcohol series and then wash them in running water for 15 minutes.
4) Block endogenous peroxidase activity by incubating in 3% hydrogen peroxide in distilled water for 5 minutes. (6ml H_2O_2 in 200ml Dist Aq)
5) Wash in water for 5 minutes.
6) In order to enhance antigen retrieval, place slides in a plastic Coplan jar, submerged in citrate buffer (Appendix II:X), and heat in a household microwave oven (600W) on 100% power for two periods of 5 minutes. Ensure that the sections remain covered at all times.
7) Leave to stand for 15 minutes.
8) Wash with TRIS buffered saline (TBS).
9) Incubate sections in normal rabbit serum (NRS), diluted 1:20 in TBS, for 10 minutes. (300μl NRS in 6ml TBS)
10) Wash with TBS.
11) Incubate with the monoclonal antibody for 1 hour.
12) Wash off excess monoclonal antibody with TBS.
13) Incubate in biotinylated-rabbit-anti-mouse serum (RxM(B)) for 30 minutes. (3μl RxM(B) in 100μl TBS)
14) Rinse with TBS.

15) Apply streptavidin for 30 minutes at 1:500 dilution. (2μl streptavidin in 1000μl TBS)
16) Wash in TBS.
17) Incubate sections with the DAB medium (Appendix II:XI) for 7 minutes.
18) Wash well in running water.
19) Lightly counterstain in Mayer's Haematoxylin (Appendix II:XIII) for 1 minute.
20) Blue in running water for 5 minutes.
21) Dehydrate, clear and mount in DPX.

APPENDIX II

SOLUTIONS AND STAINS

I) PARAFORMALDEHYDE - GLUTARALDEHYDE FIXATIVE

Stock solution

Solution A

Dissolve 22,6g of $NaH_2PO_4.H_2O$ in 100ml of distilled H_2O.

Solution B

Dissolve 25,2g of NaOH in 1000ml of distilled H_2O.

Solution C

Add 170ml of solution B to 830ml of solution A and pH to 7,4 using 1N HCl. Then add 0,025g of CaCl per 1000ml of final solution.

Procedure

Dissolve 20g of paraformaldehyde powder in 400ml of distilled H2O, by heating to 60^{o} while stirring continuously in a covered beaker to minimize evaporative loss. Add solution B dropwise until solution clears. One to five drops should be sufficient. Finally add 500ml of solution C and 100ml of 25% Glutaraldehyde in order to make up 1000ml of fixative. If necessary, pH to 7,4.

II) 0,2M PHOSPHATE BUFFER

Stock Solution

Solution A

Dissolve 3,12g of sodium dihydrogen orthophosphate in 100ml of distilled H_2O.

Solution B

Dissolve 2,83g of disodium hydrogen orthophosphate in 100ml of distilled H_2O.

Procedure

Add 9,5ml of solution A to 40,5ml of solution B and pH to 7,4.

III) OSMIUM FIXATIVE

(1% Osmium tetroxide in 0,2M Phosphate buffer)

Stock solution

Solution A

2,26g of $NaH_2PO_4.2H_2O$ in 100ml of distilled H_2O.

Solution B

2,25g of NaOH in 100ml of distilled H_2O.

Solution C

5,4g of glucose in 100ml of distilled H_2O.

Procedure

Mix 41,5ml of solution A with 8,5ml of solution B. Adjust the pH to 7,4. Add 45ml of the resultant solution to 5ml of solution C. Add 0,5g of Osmium tetroxide to final solution and store overnight prior to use in order to allow Osmium to dissolve.

IV) SPURR EMBEDDING EPOXY RESIN

Constituents

ERL 4206 (Epoxy Resin)	20g
DER 736 (Plasticiser)	8g
NSA (Anhydride Hardner)	52g
S1 (Accelerator)	0.8g

Procedure

Weigh sequentially into a glass beaker the ERL, DER, NSA and the S1. Mix thoroughly using a glass stirrer for a least 5 minutes. Store in a covered beaker in the freezer until required.

V) 8% SATURATED SOLUTION OF URANYL ACETATE

Constituents

Uranyl Acetate	8g
Distilled H_2O	100ml

Procedure

Weigh out the uranyl acetate and add it to the water contained in a beaker. Mix thoroughly using a stirrer bar until the solution appears saturated. Store in an opaque glass bottle at $4^{o}C$ until required.

VI) REYNOLD'S LEAD CITRATE STAIN

Constituents

Lead nitrate	1,33g
Sodium citrate	1,76g
1N Sodium hydroxide	8ml
Distilled H_20	42ml

Procedure

Add the lead nitrate and sodium citrate to 30ml of distilled H_2O. (A heavy white precipitate will be formed) Shake vigorously at minute intervals for thirty minutes. Add the sodium hydroxide and dilute to 50ml with the remaining distilled H_2O. (The precipitate will dissolve) Store at $4^{o}C$ and filter prior to use.

VII) TRIS BUFFER FOR RIA

(0,01M Tris-HCl, 0,0015M EDTA, 10% Glycerol, 0,001M Monothioglycerol)

Constituents

Tris (hydroxymethyl) methylamine	1,2114g
EDTA (ethylenediaminetetracetic acid)	0,5584g
Glycerol	100ml
Monothioglycerol	5μl/50μl stock

Procedure

Add all constituents (except the monothioglycerol) together in distilled H_2O, made up to 1000ml. Cool buffer to $4^{o}C$. Using 12N HCl, pH to 8. Add 5μl of monothioglycerol per 50μl of stock solution just prior to use.

VIII) DEXTRAN COATED CHARCOAL FOR RIA

Constituents

Norit A (Activated charcoal)	2,5g
Dextran	0,025g
Tris (hydroxymethyl) methylamine	1,2114g

Procedure

Add all constituents together in distilled H_2O, made up to 1000ml. Cool to 4^oC. Using 12N HCl, pH to 8.

IX) TRIS BUFFERED SALINE FOR ICC

(0,05M Tris-HCL)

Constituents

Tris (hydroxymethyl) methylamine	24,6g
HCl (1N)	148ml
NaCl	28,8g

Procedure

Add all constituents together in distilled H_2O made up to 4000ml. Using 2N NaOH, pH to 7,6.

X) CITRATE BUFFER FOR ICC

Constituents

Citric acid	2,1g
Distilled H_2O	900ml

Procedure

Weigh out citric acid and add to 900ml of distilled H_2O. Using 2N NaOH, pH to 6,0 and make up to 1l.

XI) DAB FOR PEROXIDASE DEVELOPMENT

Constituents

Diaminobenzidine tetrahydrochloride(DAB)	0,005g
DAB buffer	5ml
1% H2O2	50µl

Procedure

Add the above constituents together in a test tube and mix well. Use immediately.

XII) TRIS BUFFER FOR DAB

Constituents

Tris(hydroxymethyl) methylamine	24,228g/l H_2O
HCl(0,1N)	19ml

Procedure

Combine 12ml of 0,2M Tris with 19ml of 0,1N HCl. Make up to 50ml with distilled H_2O. pH to 7,6.

XIII) MAYER'S HAEMATOXYLIN FOR ICC

Constituents

Haematoxylin	1g
Distilled H_2O	1000ml
Potassium alum	50g
Citric acid	1g
Chloral hydrate	50g
Sodium iodate	0,2g

Procedure

Dissolve the haematoxylin, potassium alum and sodium iodate in the distilled H_2O by stirring while gently heating. Then add the Chloral hydrate and citric acid. Bring to the boil for 5 minutes, cool and then filter.

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