

PROGESTERONE, ESTRADIOL AND THEIR RESPECTIVE RECEPTORS

IN LEIOMYOMA AND ADJACENT NORMAL MYOMETRIA

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BY

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*A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE*

*OF*

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**DEDICATION**

To my wife Miranda

my children Camille, Lionel and Faith

for their love, patience and support.


DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.



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**ABSTRACT**

Scientific work has generated a concensus on the involvement of sex steroid hormones and their respective receptors in the development of uterine fibroids, but there are controversies as to the levels of these hormones and receptors in this disease. The present effort was directed towards resolving this controversy and to provide information regarding the disease in the black (negroid) population in Kenya.

Specimens of uterine leiomyomata and the adjacent normal myometria were collected from twenty patients undergoing hysterectomy, at the Kenyatta National Hospital Nairobi, for histological examination and analysis of progesterone ( $P_4$ ), estradiol ( $E_2$ ), estrogen receptor (ER) and progesterone receptor (PR) levels.

The tissues for hormonal and receptor measurements were homogenized and centrifuged at 27,000 x g for 40 minutes to yield a supernatant (cytosol). The cytosolic fluids were assayed for total protein as well as their binding activity to estradiol and progesterone tracers. Fractions of cytosol were incubated separately with [(2,4,7- $^3H$ )Estradiol] and [17 $\alpha$ -Hydroxyl(1,3,6,7- $^3H$ )]Progesterone at 4°C for 48 hours. Using centrifugation bound and unbound ligand were then separated by charcoal absorption. The supernatant was counted in a Beta Liquid Scintillation Counter. The amount of bound hormone per mg protein was calculated.

Estrogen and progesterone receptors (ER & PR) were indirectly determined by calculating the amount of bound hormone per mg cytosolic protein while the estradiol and progesterone levels were determined by radioimmunoassay (RIA). The results showed that the adjacent normal myometria contained significantly higher levels of  $E_2$  (181.1 % :  $P < 0.001$ ) and  $P_4$  (240.6 % :  $P < 0.001$ ) compared to the leiomyomata. The total  $P_4$  in the uterine tissues i.e fibroid and myometrium was higher (628.4 %) than total  $E_2$  in the same tissues. On the contrary, the uterine leiomyomata contained higher levels of ER (147.6 % :  $P < 0.001$ ) and PR (178.7 % :  $P < 0.001$ ) compared to the myometria. The total ER was higher (180.5 %) than total PR.

It was therefore concluded that women in the black population in Kenya have higher levels of  $E_2$  and  $P_4$  in the adjacent normal myometrium compared to the leiomyoma. On the other hand, ER and PR concentrations were higher in the leiomyoma compared to the adjacent normal myometrium. In the same population total ER concentration i.e ER in both fibroid and myometrium were higher than total PR in the same tissues, whereas, total  $P_4$  levels were higher than  $E_2$ . It is postulated that relative proportions of  $E_2$ ,  $P_4$ , ER and PR in individual patients uterine tissue may be important in the pathogenesis of fibroids in the black population in Kenya. It is further suggested that treatment and management of the problem should involve manipulations of the sex steroids and their receptors.

However, research should continue in order to indentify a nonsurgical means of treating the disease. This would be an important public health initiative especially in the black population where the incidence of fibroids is higher, surgical complications commoner and childbearing the center of matrimonial harmony. While hoping for such a breakthrough the importance of early diagnosis and the detection of high risk groups should be emphasized in the clinical management of this disease since the current drug treatment modality only reduces uterine size.



## CHAPTER ONE

### LITERATURE REVIEW

#### 1.1 INTRODUCTION

Uterine leiomyomata (fibroids) are the most common pelvic tumours in women, arising in the muscle wall of the uterus (Steward, 1969). They are circumscribed benign tumours composed mainly of smooth muscle but having fibrous connective tissue elements, hence the name fibroid. The name leiomyoma is commonly used since it designates neoplasm of smooth muscle origin. Other descriptive names to portray the tissue components of the new growth also used are myomas, fibromyomas and fibroleiomyomas (Brewer and DeCosta, 1967). The tumour is demarcated from surrounding muscle, which may flatten out to form a pseudo capsule. Fibroids first arise during the child-bearing period of life, do not arise during menopause. The successful fertility of women is reduced by approximately 30 per cent if fibroids are present (Steward, 1967).

The most common and characteristic symptom of these tumours is abnormal uterine bleeding characterized by an excessively long menstrual period or by excessive bleeding during the normal period. These symptoms vary greatly depending on the size, number and location of the leiomyoma (Russell, 1977). Approximately 25 to 35 percent of women with leiomyomas are sterile. Sterility as such is

not a common complaint, the infertility of most patients with leiomyoma does not lie in their ability to become pregnant, but rather in maintaining a pregnancy when it occurs (Russell, 1977).

Uterine leiomyoma are not only the commonest neoplasms in the female pelvis but also the commonest indication for hysterectomy, accounting for one third of these operations (Russell, 1977). Not counted in these statistics are additional surgical procedures, including myomectomy and uterine curettage for abnormal uterine bleeding. One quarter of women of reproductive age are diagnosed with leiomyomata uteri, giving rise to a large population of women at risk for pain and abnormal uterine bleeding. Identification of non-surgical means of treating this disorder is an important public health initiative (Russell, 1977). In this regard, the use of gonadotropin releasing hormone-agonist (GnRH-a) in the treatment of symptomatic leiomyomata has been investigated intensively (Goland, Bukovsky, Schneider, Ron-El et al. 1989 ; Friedman, Rein, Harrison-Atlas, Garfield, Donbit, 1989). These agents decrease circulatory levels of estrogens, which are believed to be necessary for growth and perhaps maintenance of the neoplastic cells and extracellular matrix of the leiomyomata (Russell, 1977). The tumours appear to occur at a higher incidence among the black (negroid) population (Collins, Levin and Savage, 1980 ; Sadan Van Iddekinge and Van Gelderen, 1987).

The tendency of uterine fibroids to arise during reproductive life, to grow during pregnancy and to regress post menopausally implicates steroid hormones (estrogen and progesterone) as factors

in the etiology and pathogenesis of the tumour (Langdon and Sheldon, 1963). Research work done so far has implicated steroid hormones and steroid hormone receptors (estrogen and progesterone receptors) in the development of the disease, and this has revolutionized the management of uterine fibroids. Currently some of the management protocols involve the use of compounds which decrease circulating estrogens and progesterone, for example leuprolide acetate depot (Friedman et al. 1989). This new method of management is based on the background knowledge that fibroids are extremely sensitive to endogenous steroids, indicating the presence of high levels of steroid hormone receptor concentrations in the tissue (Van Leusden, 1986).

However, various research work done so far are at variance regarding the concentrations of steroid sex hormones and their receptors in the fibroid tissue and the myometrium (Pukka, Kontula, Jane, Vihko, 1976 ; Pollow, Sinneck, Boqueri, Pollow, 1978 ; Tamaya, Motayama, Ohomo, Ide et al. 1979 ; Wilson Yang, Rees, 1980 ; Jorge, Edgrad, Jacques, Christine et al. 1990 ; Porgieter Magagane, Bester, 1995). It is against the background of this controversy that this research work was formulated. Thus, this study was designed to determine the levels of estradiol and progesterone; estrogen and progesterone receptor status in the uterine leiomyomata and the adjacent normal myometrium. It is hoped that the results will be of value in expanding our knowledge with the view of improving the clinical management of these tumours.

Uterine leiomyomata have been identified to be the most frequently occurring tumours of the uterus; occurring in 20 to 25 percent of women in the reproductive age group (Healy, Shakelton, Downing et al. 1989). Tindell (1987) describes uterine fibroids as rare before the age of 20 years, and states that they are more common in nulliparous or relatively infertile women. He states that women of certain races, notably the negro race are especially prone to develop fibroids, and no apparent cause for this association has been identified.

In Africa, the United States and the West Indies fibroids seem to be commoner in black women, in whom they develop earlier and grow larger than in Caucasians (Lawson and Stewart, 1967). These workers suggested the probability of a true racial difference, though it may also be influenced by other yet unidentified factors. Fibroids are frequently associated with primary and secondary infertility as was the case in 76 percent of a group studied in Jamaica (Lawson and Stewart, 1967).

In the United States, the tumours occur most frequently in Jewish and African-American patients (Lawson and Stewart, 1967). Approximately 50 percent of black females develop leiomyoma at some stage in their lives, and it has been suggested that this phenomenon might be related to the higher incidence of keloid formation in the black race (Russell, 1977). However, it has been reported that certain genetically uncontaminated African tribes are

almost totally free of this particular tumour (Russell, 1977). The latter literature did not state where the study was done nor the identity of the tribes concerned.

The tumours may occur singly but are usually multiple and as many as 100 or more have been found in the same uterus (Russell, 1977). Russell (1977) also states that new tumours may continue to appear throughout the reproductive life of the individual and their sizes may range from microscopic to huge, sometimes filling the entire abdominal cavity. About 98 percent of leiomyoma occur in the uterine body and 2 percent occur in the cervix. Other sites include the vagina, vulva, fallopian tubes and the round ligaments (Russell, 1977).

The growth rate of leiomyoma is characteristically slow. The increase in size over a six-month period is scarcely noticeable in the nonpregnant patient, but may be accelerated during pregnancy because of the increased blood supply and cellular growth caused by the increased level of estrogen (Russell, 1977). In the absence of pregnancy, an accelerated rate of growth may indicate degenerative or rarely sarcomatous change, especially after menopause (Russell, 1977). These tumours rarely occur before menarche and never develop after menopause although large tumours have been found in women in their early twenties and huge uncalcified tumours have been found in patients in the 75 to 85 year age group (Russell, 1977). Comparable but not identical tumours have been described in the monkey, the pig, the whale and the cow (Lawson and Stewart, 1967).

### 1.3.1 Etiology.

Factors that have been associated with the development of fibroid include age, parity, mechanical stresses, ovarian function, racial and genetic factors (Jeffcoate 1957). Other theories that have been postulated include the Meyer,s theory (Russell, 1977) and the development from embryonal cells in the walls of the uterine blood vessels as explained below according to Blaustein (1977).

Age: It has been observed that fibroids are rare before the age of 20 years but are to be found if only as tiny tumours, in approximately 10 percent of women over the age of 40 years. They mostly cause symptoms between the ages of 35 and 45 years but probably exist in microscopic form before the age of 30 years.

Parity: Fibroids are more common in nulliparous or relatively infertile women but it is not known whether sterility causes fibroids or vice versa, or whether both conditions have a common cause. The general view is that the uterus which is deprived of pregnancies consoles itself with fibroids or, as the old adage put it, 'fibroids are the reward of virtue, babies the fruit of sin' (Blaustein, 1977).

Racial and genetic factors: Women of certain races, especially the Negro, are especially prone to develop fibroids. Also, irrespective

of races these can have a familial incidence.

Mechanical stresses: One concept put forward explains fibroids as fibromuscular reactions to mechanical stresses in myometrial wall. These stresses operate mainly in a uterus not protected by pregnancy. This theory can account for the sites of fibroids (along the lines of stress) and their association with nulliparity, but it is not widely accepted.

Ovarian function: It is often suggested that excessive estrogen stimulation causes fibroids but the evidence is unconvincing. These tumour do not significantly atrophy at the climacteric, as was suggested at one time. Moreover, they sometimes arise after menopause - even after bilateral oophorectomy at an early age.

Meyer's theory: This theory postulated that fibroids arise from immature smooth muscle cells and cell nests. The originating cell in this case would be located in the myometrium and would be undifferentiated from mesenchymal myometrial cells.

Vascular leiomyomata: Commonly referred to as angiomyomas and are characterized by an associated number of blood vessels relative to the amount of smooth muscle. It is not clear whether the vessels in these lesions are proliferating as part of a neoplastic process or whether they simply present a hyperplastic change. Vascular leiomyomata are usually submucosal in location, although they may

be intramural or subserosa. The cut surface of the tumour shows numerous small, thin-walled, well-formed blood vessels.

Associated conditions:

Anovulation, combined with sustained stimulation of the uterus by estrogen uninterrupted by corpus luteum formation and production of progesterone, may create a hormonal imbalance leading to growth of leiomyomas (Russell, 1977). The same author states that this mechanism may be set in motion during early adolescence when anovulation is common and since leiomyomata are found in patients at an average age of 37 years, hormonal stimulation over a period of 20-25 years seems necessary to cause symptoms before the tumours are clinically apparent. Because of the temporal association of leiomyoma growth with physiological states in which circulating levels of ovarian steroids are relatively high, both estrogen and progesterone have received attention as mediators of leiomyoma development (Buttran and Reite, 1981). Langdon and Sheldon (1963) also linked the etiology of the disease to estrogen stimulation and proposed that there is a definite age of incidence, and that the growths are not found before the onset of puberty.

Diseases commonly and possibly significantly associated with fibroids are follicular cysts of the ovary, endometrial hyperplasia, endometrial carcinoma and endometriosis (Tindall, 1987).



### 1.3.2 Pathology.

Gross pathology: Leiomyomas are encapsulated in the sense that they do not invade adjacent tissue, although no real capsule exists (Russell, 1977). The pseudo capsule is composed of fibrous and muscle tissue of the myometrium that has been compressed by the tumour, and since the vasculature is located in the periphery, the central part of the tumour is highly susceptible to relative ischaemic changes (Russell, 1977). Although the tumours are typically firm and spherical some are irregularly lobulated, and on cut surface they are smooth, firm and usually pinkish-white; and their colours may vary with the degree of vascularity (Russell, 1977). The surface typically has a trabeculated, whorl-like appearance that has often been compared to watered silk and in large tumours this pattern may be blotted out by the common hyaline degeneration (Russell, 1977).

Microscopic pathology: Microscopically, the leiomyoma is composed of bundles of smooth muscle fibers arranged in a whorled fashion and high-power examination reveals spindle-shaped cells with elongated cigar-shaped nuclei of uniform size and staining quality (Russell, 1977). Although leiomyomas are composed essentially of smooth muscle cells, they almost invariably contain some connective tissue elements. In some instances, fibrous tissue cells may predominate and pathologic designations such as "fibromyoma" or "myofibroma" are sometimes employed, depending on the predominant

element in the tumour. The cellular type of leiomyoma is characterized by an abundance of muscle cells with almost no fibrous connective tissue stroma (Russell, 1977).

Degenerative changes: Leiomyomas may undergo degeneration, and rarely sarcomatous change. Most of the degenerations occur due to the presence of circulatory impairment in the capsule caused by an acute disease or exacerbation of a chronic disease (Russel, 1977). The different types of degeneration that have been described include:

(a). Hyaline degeneration:- Usually it is caused by an overgrowth of the fibrous elements with an ultimate hyalinization of the fibrous tissue and eventually calcification. Microscopically, hyalinized areas appear diffusely-pink stained, standing out sharply from the preserved cellular muscle tissue (Russell, 1977).

(b). Cystic degeneration:- Cystic cavities are usually thought to be a result of liquefaction following hyaline degeneration and may occasionally be a sequel of necrosis (Russell, 1977).

(c). Red degeneration; necrobiosis:- This is mostly seen during pregnancy and puerperium but can occur at other times. The tumour becomes soft and homogenous or necrotic, especially in the centre, and is diffusely stained red or salmon pink.

Histologically the degenerative area appears structurally and poorly stained, and there is evidence of thrombosis in some of the vessels. The pathogenesis is obscure but the initial change appears to be one of subacute necrosis which is presumably caused by an interference with blood supply (Tindall, 1957).

(d). Fatty degeneration:- Sometimes in association with partial necrosis, a fibroid contains fat. At a later stage in the process is the deposition of calcium, first in the form of calcium soaps. The calcium may be diffused throughout the tumour, a change which ultimately produces a 'wombstone', or it may have a peripheral 'eggshell' distribution. The latter happens when the centre of the fibroid is completely avascular and necrotic, the former when the persistence of some circulation permits multifocal deposits (Tindall, 1957).

(e). Sarcomatous change:- It is generally believed that sarcoma cells originate by direct heteroplasia from smooth muscle and connective tissue cells. The sarcomatous areas are generally recognizable by their loss of fibrous structure, resulting in a softer, homogenous, fleshy appearance grossly (Russell, 1977). The histological characteristics of leiomyosarcoma, in contrast to leiomyomata, include increased cellularity, a change in appearance of nuclei from thin elongated structures to plump spindle-shaped nuclei, greater

variability in size and staining properties, prominent nuclei and increased mitotic figures. The cytoplasm is eosinophilic and poorly defined. Marked anaplasia and tumour giant cells may be present (Blaustein, 1977).

### 1.3.3 Pathogenesis.

Research work that has been done on the pathogenesis of leiomyomata strongly suggest that they are unicellular in origin, and although factors responsible for the neoplastic transformation are not presently known, it has been suggested that estrogen may influence the rate of growth of the tumour (Townsend, Sparkers, Baluda and Mclellanda, 1970). Clinically, it has been reported that estrogen deprivation will result in shrinkage of leiomyomata pointing to the importance of estrogen receptors (ER) in the pathogenesis of uterine leiomyomata (Grainger, Carol, Harbison et al. 1993) .

The work done to determine the etiology and pathogenesis of this disease has not been limited to humans. Nelson (1937) and Lipshutz (1942) elicited leiomyomata in guinea pigs subjected to prolonged estrogen stimulation. However, Segaloff, Weed, Sternberg and Parson (1949) demonstrated that guinea pig tumours are not analogous to human tumours and differ histologically from those in human.

In 1980, Wilson, Yang and Rees reported significantly higher concentration of ER in the leiomyomata than the adjacent

myometrium. Hence, the pathogenesis of fibroids may be centered around steroid hormone (estrogen, progesterone) and steroid hormone receptors (ER and PR).

#### 1.4 REPRODUCTIVE IMPLICATIONS OF UTERINE FIBROIDS

Leiomyomata are frequently asymptomatic and have been reported to reach the size of a full-term pregnancy with no apparent symptoms (Russell, 1977). Twenty to fifty percent of fibroids are estimated to cause symptoms and the severity of these symptoms will depend upon the number, the size, and location of the tumours (Ingersol, 1963; Hunt and Wallaech, 1974; Babaknia, Rock and Jones, 1978). Submucous and polypoid tumours show the most frequent symptoms, while the subserous type show the least (Fig 1). Symptoms do not appear after menopause unless significant degenerative changes have occurred. (Russell, 1977).

The most common and characteristic symptoms is abnormal uterine bleeding, commonly seen in fibroids located in the submucosa, characterized by excessive bleeding during the normal period or excessively long menstrual periods. Polypoid tumours when present cause continuous bleeding or spotting associated with pain (Jones and Jones, 1981). Habitual abortion may occur when the pregnancy co-exists with multiple leiomyomas and this may be due to the fact that when the conceptus vies for space with the existing tumour, the conceptus is usually expelled (Russell, 1977). Abortion

could also occur as a result of failure of the uterus to expand, poor placentation or uterine distortion. Other symptoms include extra-uterine pregnancies, spontaneous abortion, pre-mature labour, difficult labour resulting in stillbirths and neonatal deaths. Statistically the successful child bearing activities of a woman are reduced by approximately 30 percent if fibroids are present (Stewart, 1969). Work done by Buttram and Reite (1981) revealed that 29 percent of patients with leiomyomata experienced menorrhagia and that 1063 pregnant women studied 441 (41 percent) ended in spontaneous abortion.

The disturbances of childbearing that can be ascribed to leiomyomas are more often the result of mechanical interference with the delivery of the fetus than with the ability to conceive (Russell, 1977). By their size and/or location myomas may produce an obstruction to the normal vaginal delivery of a term fetus and the tumours that are most likely to cause the latter are those located in the lower portion of the uterus, in the cervix, and the posterior cul-de-sac (Fig 2). Further, the efficiency of uterine contractions necessary for the expulsion of the fetus may be interfered with in the presence of fibroids that occur in the myometrium. In addition, faulty separation and delivery of the placenta and post partum hemorrhage may be a direct result of the leiomyomas, and malposition of the fetus may be due to these tumours (Russell, 1977).

Leiomyomas may cause mechanical interference with uterine implantation of a fertilized ovum. Also, it is possible that an

endometrium whose vascular system is markedly distorted by the tumours may offer a poor implantation site to a fertilized ovum that has found its way into the uterine cavity (Brewer and DeCoster, 1967). When implantation does occur there may be an increased incidence of abortion in patients with leiomyomata, particularly if the tumours are submucous or polypoid (Brewer and DeCoster 1967). Nevertheless, it is common to find multiple tumours which do not interfere with the maintenance and normal completion of a gestation ( Brewer and DeCosta, 1967).

Hysterosalpingography done on patients with fibroids has shown uterine cavities with both tubes being obstructed at the cornual end resulting in infertility. Cases of uterine inversion due to prolapse of submucous fibroids have also been reported ( Russell, 1977).

11. 197



11. 197



FIGURE 1: The distribution, extension and microscopic appearance of uterine leiomyomata.

(Adopted from Textbook of Gynaecology by Russell, 1977)

1 = Cervical.

2 = Intraligamentary.

3 = Subserous.

4 = Interstitial (intramural).

5 = Pedunculated; subserous.

6 = Subserous; displacing tube.

7 = Pedunculated; submucous.

8 = Submucous.

9 = Pedunculated; submucous, protruding through external os (polyp).

FIGURE 1.

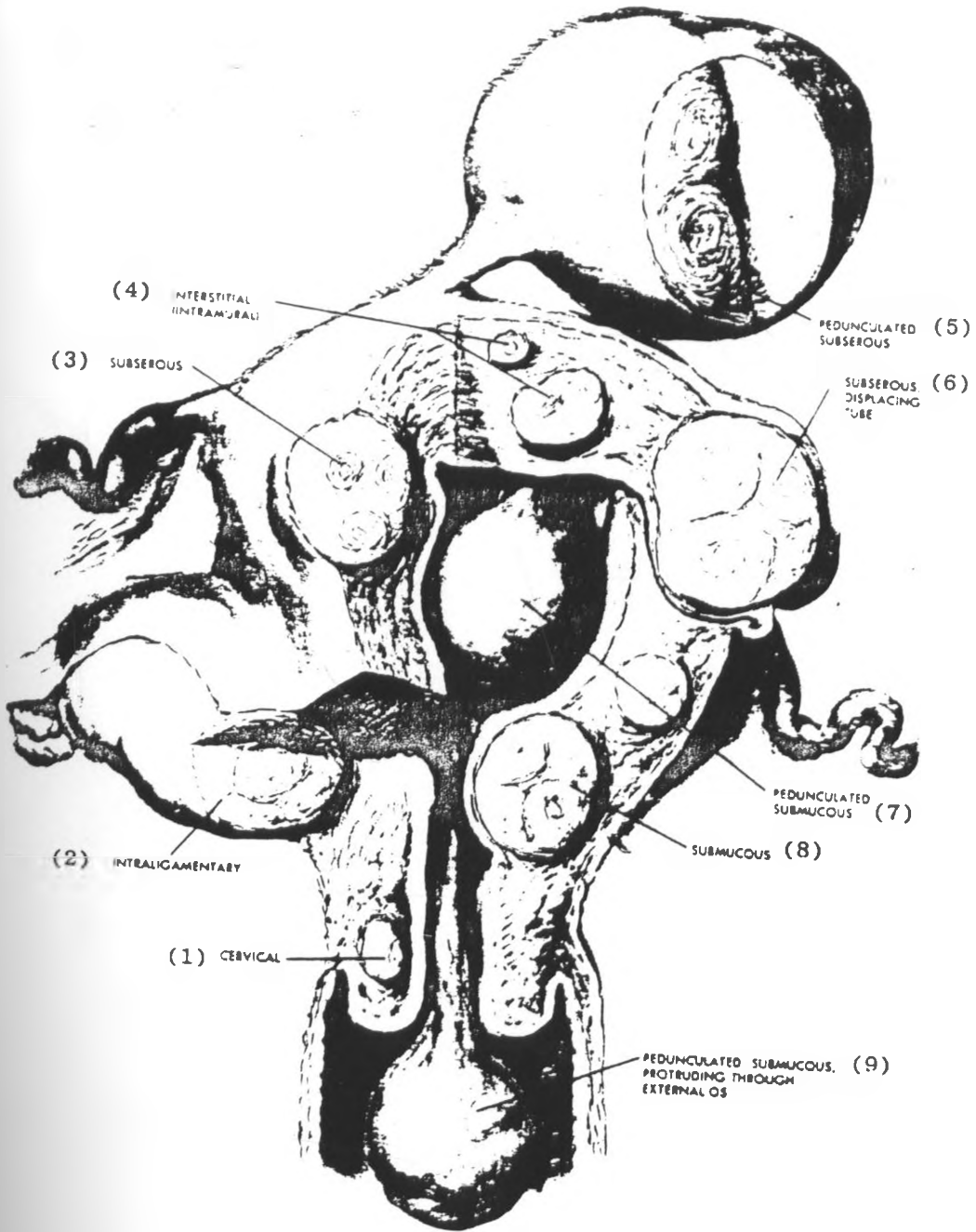


FIGURE 1  
Inclination  
of uterus

FIGURE 2  
Inclination  
of uterus



(a)



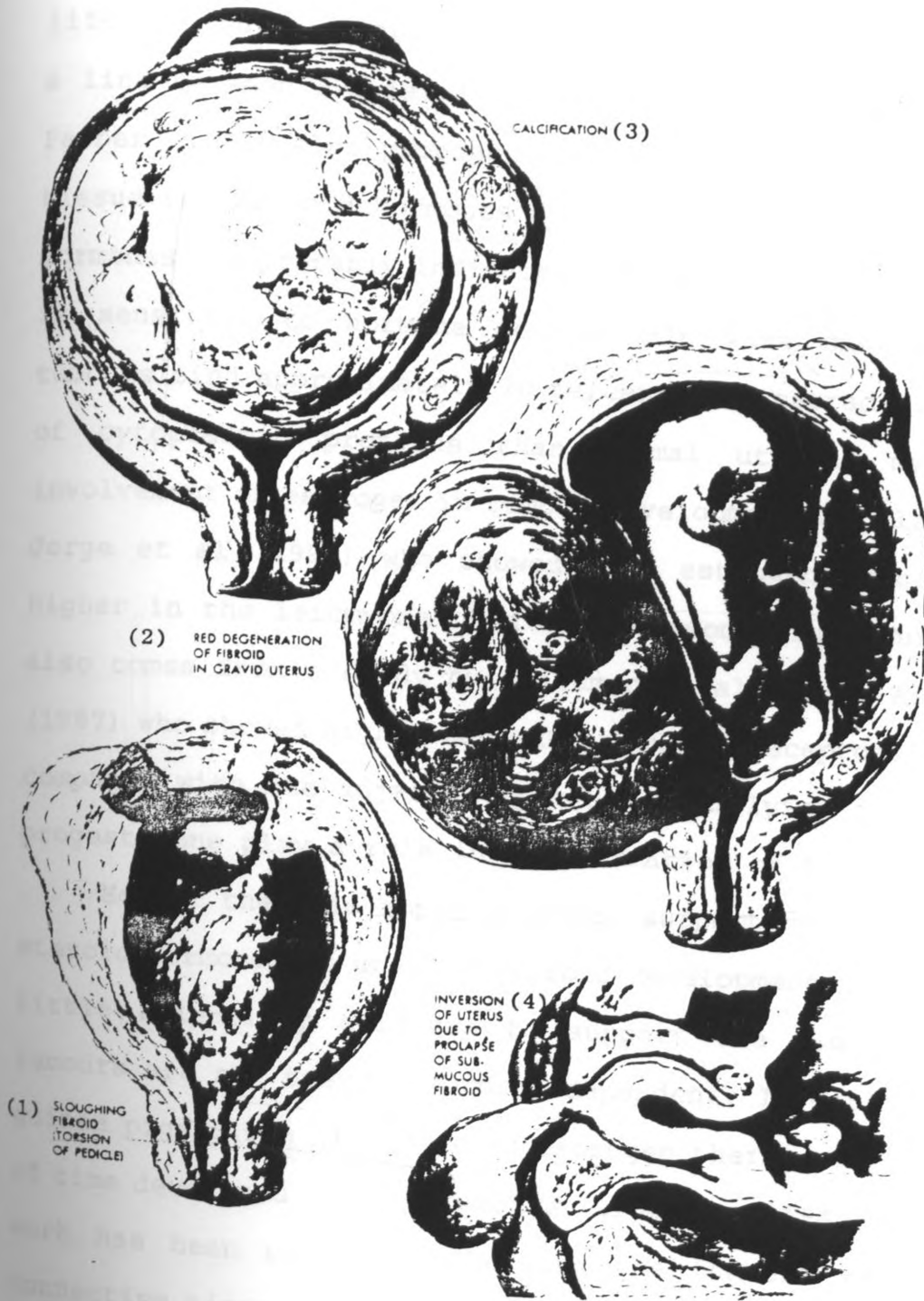
(b)



FIGURE 2: Types of degeneration of leiomyomata including calcification, torsion, inversion of the uterus and accompanying pregnancy.

(Adopted from Textbook of Gynaecology by Russell, 1977)

- 1 = Sloughing fibroid (torsion of pedicle).
- 2 = Red degeneration of fibroid in gravid uterus.
- 3 = Calcification.
- 4 = Inversion of uterus due to prolapse of submucosal fibroid.



## 1.5. STEROID HORMONES AND RECEPTORS INVOLVED IN LEIOMYOMATA

Leiomyomata are usually present in the childbearing period of life and regress after menopause, thereby suggesting that there is a link between ovarian function and tumour growth (Farber, 1972). Farber (1972) also proposed that the specific high affinity of a tissue to steroid hormones determines its capacity to retain the hormones and probably indicates a degree to which the target tissue is sensitive to hormone action. His work showed that fibroid tumours bind approximately 20 percent more estradiol per milligram of cytoplasmic proteins than normal uterine myometrium. The involvement of estrogen in tumour development was also supported by Jorge et al (1990) who showed that estrogen concentrations are higher in the leiomyoma than in the myometrium. Further evidence also comes from a study by Friedman et al (1989) and Sadan et al (1987) who showed higher concentrations of receptors in the tumours compared with the adjacent myometrium implying that estrogen and progesterone play a role in the formation of the leiomyomata.

Hence, there is ample evidence supporting the involvement of steroid hormones in uterine fibroid development. However, there is little scientific evidence to support the suggestion that the tumours are sex steroid-hormone dependent. In earlier experiments guinea pigs given high-dose of estrogen therapy over a long period of time developed uterine tumours (Lipschutz, 1942). However, this work has been criticized because the tumours were composed of connective tissue and were not leiomyomata (Jorge et al. 1990). It

is also worth noting that there are no animal models of fibroids and therefore experimental evidence on the steroid dependence of fibroids is limited to in vitro studies (Vollenhoven, Lawrence and Healy, 1990).

With the lack of animal models, treatment modalities are being used to determine the involvement of steroid hormones in fibroids. In this regard the use of GnRH-a to shrink the fibroid tissue is now common and the mechanism of action is considered to be via reduction of serum estradiol concentration to postmenopausal levels (Fernandez-Montoli, Diez-Gibelt, Samaniego et al. 1995). Scialli and Jestila (1995) suggested that GnRH-a may act in shrinking fibroids by the reduction of luteal phase progesterone levels. This has been supported by the fact that treatment with GnRH-a also results in anovulation and loss of the luteal phase rise in serum progesterone levels (Fernandez-Montoli et al. 1995). Interestingly, in a study comparing treatment of fibroids with GnRH-a to treatment with an analogue plus medroxyprogesterone acetate, uterine volume significantly decrease in patients taking the analogue alone but not in those also taking progesterone (Friedman et al. 1988). It is therefore possible that GnRH-a may act in shrinking fibroids by reduction of luteal phase progesterone levels (Scialli and Jestila 1995).

In 1995, Harrison-Woolrych and Robinson reported higher levels of epidermal growth factor messenger ribonucleic acid (EGF mRNA) in fibroids compared with myometrium, but only in the secretory phase of the cycle, when progesterone is the influential hormone. From

these results they suggested that progesterone may be important in fibroid growth. Further evidence came from clinical study in which 10 patients with fibroids were treated with the anti-progesterone RU486 for 3 months. Leiomyomata volume decreased by 49 per cent (average) and uterine tissue (fibroid and normal myometrium) examined after hysterectomy showed significantly decreased progesterone receptor content in the treated patients ( Murphy, Kettel, Morales, Roberts and Yen, 1993). A study by Harrison-Woolrych and Robinson (1995), showed an increase in uterine fibroid size secondary to treatment with high-dose progestogen. Concurrent treatment with antiestrogen (tamoxifen) did not prevent the enlargement occurring. Tamoxifen also may have up-regulated progesterone receptors on the leiomyomata, enhancing the effect of megestrol acetate. After removal of megestrol acetate shrinkage of the fibroid occurred within one month. They, therefore, suggested that the steroid dependence of leiomyomata needs further investigation but treatment with anti-progesterone may be important in the future management of these tumours.

Results from a study by Potgieter et al (1995) showed that plasma levels of estrogen and progesterone, in both control group and experimental groups with leiomyomata were essentially similar. These results supported published observations that patients with leiomyomas exhibited normal circulatory levels of estrogen and progesterone (Sadan et al. 1990).

There appears to be little consensus regarding the quantitative distribution of estrogen receptors (ER) and



progesterone receptors (PR) in normal and myomatous uterine myometria. In 1980, Wilson et al, studied the ER in the tumour and reported a significantly higher concentration of ER in the leiomyomata than in the normal myometrium of the same uterus. While in the same year Buchi and Keller (1983) reported that no differences existed between the concentration and the hormonal modulation of steroid receptors in normal and myomatous myometria. Earlier studies by Pukka et al (1976); Pollaw et al (1978) and Tamaya et al (1979) did not show a difference between the ER concentration of leiomyomata and adjacent myometrium. The most recent work done to examine the status of ER and PR is that by Potgieter et al (1995) who reported significantly higher levels of ER and PR in the myomatous uteri as compared to normal myometria of control groups. The latter workers, therefore, concluded that the development of leiomyomata is probably associated with changes in the relative proportions of ER and PR, which in turn may affect the production and secretion of paracrine and/or endocrine factors causing aberrant proliferation of smooth muscle cells, leading to the formation of benign fibroids.

Clinically, Gaigner et al (1993) observed that estrogen deprivation will result in shrinkage of leiomyomata. Their finding supported the importance of ER in the pathophysiology of the tumour, thus, suggesting that the suppression of leiomyomata growth by GnRH-a is mediated by a decrease in estrogen receptors.

The pathways that have been described for the action of steroid hormones include an influence on the cell membrane, most clearly documented for the effect of progesterone on the oocyte maturation in fish and amphibian, steroid conversion of the native receptor to a functional transcription factor that enhances expression in target genes, and indirect effects on the actions of peptide growth factors (Jenson, 1995).

The transcription pathway is the mechanism of steroid hormone action that has been well described, and it involves a sequence of events in which the steroid binds to the native receptor to disrupt a characteristic conglomerate of associated proteins and free the receptor to undergo phosphorylation and react, in dimeric form and possibly together with other factors, with the hormone-response elements of the target genes (Jenson 1995).

It is pointed out that, by interfering with any of the events in the above pathway a substance can function as a hormone antagonist. For all classes of steroid hormones, substances are known that antagonize or prevent biological action, presumably by competing with the hormone for binding to the receptor (Jenson, 1995). Although association with the antagonist usually causes disruption of the native receptor conglomerate, subsequent steps in the reaction sequence do not proceed normally. The basis for this deficiency is not entirely clear, but it is assumed that the conformation of receptor bound to an antagonist is different from

that bound to an agonist, so that its participation in one or more critical events, such as dimerization, phosphorylation, binding to response elements, or transactivation of the target genes cannot take place (Jenson, 1995). Especially curious are the so-called type I anti-estrogens, such as tamoxifen, which act either as agonist or antagonists, depending on the species and the dose administered. This is in contrast to the type II or "pure" anti-estrogens, which show only antagonism. This present scientific understanding of the mechanism of action of steroid hormones and steroid hormone antagonists is now of immense clinical importance (Jenson, 1995).

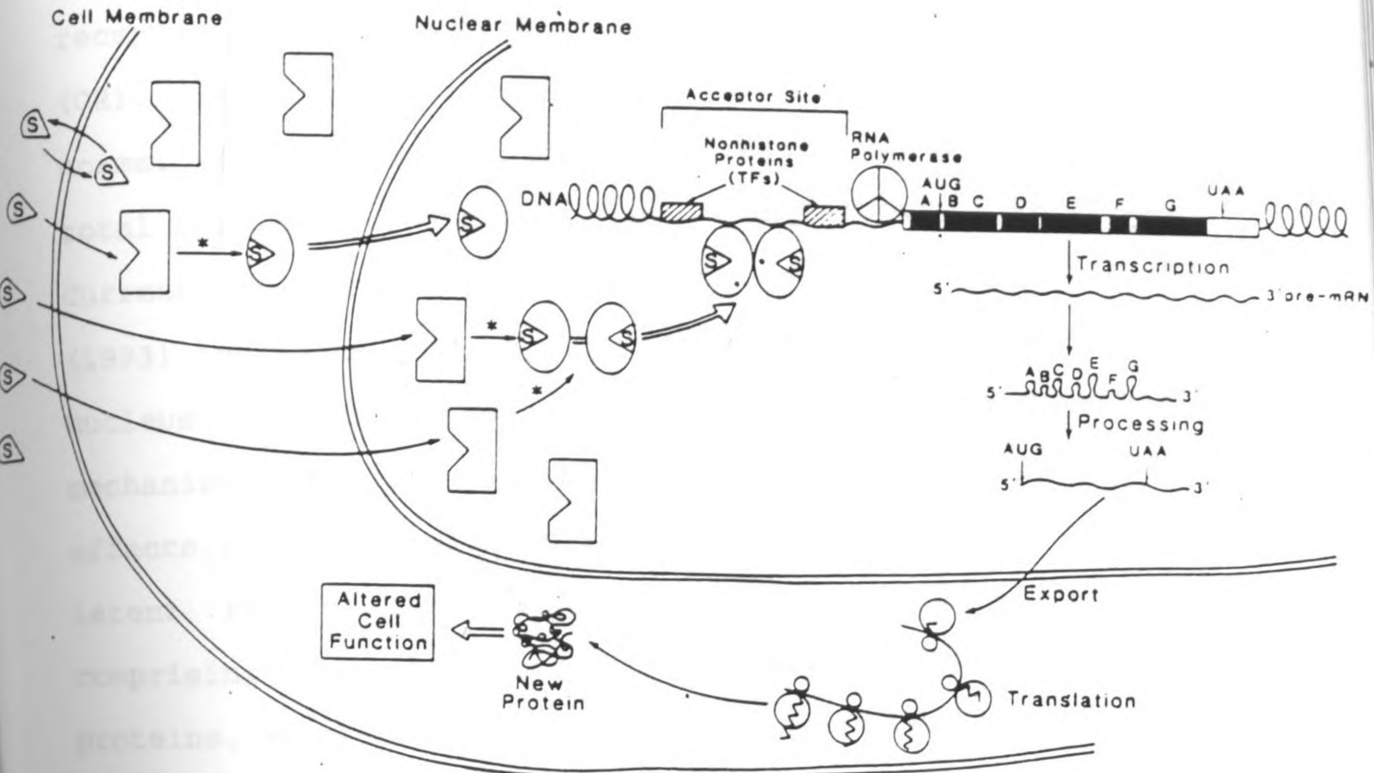
In the past, the possibility of reducing undesirable actions of steroid hormones was limited either to prevention of their formation, through the excision of hormone producing organs or blockage of the hormone biosynthesis, or, less effectively, by administering large amounts of other types of active hormonal agents. The advent of antagonists that actually can prevent hormones from acting in their target cells has provided a novel approach to endocrine regulation and opened a new era in clinical endocrinology. At present antagonists find their most extensive use in the palliative treatment of hormone-dependent tumours and in the control of reproductive processes (Jenson, 1995).

The current understanding of the biochemical pathway of steroid hormone action in the cell can be summarized briefly as follows (Fig 3.1). The hormones are secreted from their respective endocrine glands into the blood stream, where they circulate,

mostly bound (95 per cent) to plasma transport proteins, which provide a reservoir for steroid supply to cells. The free steroids diffuse into the cells and combine with specific receptors present in the target cells in which they will exert their functions. The receptor for estrogens (e.g estradiol) exists primarily in female-specific target cells such uterus, vagina, breast, and brain. After binding tightly to their specific receptors, the steroid hormones cause the receptor to undergo a conformational (allosteric) change in structure, which converts the receptors from an inactive to an active conformation. At this point the receptors have the capacity to bind to the regulatory element of the genes and activate (or suppress) their function. If a gene is activated, for example, the enzyme RNA polymerase transcribes the information in the gene into messenger ribonucleic acid (mRNA), an intermediate molecule that carries the information to the cytoplasmic compartment of cells. There the information is again decoded (translated) on structures termed ribosomes, which produce the appropriate protein product specified by the gene in question (Yen and Jaffe 1991).

Fig. 3.1

Molecular pathway of steroid hormone action. TF, transcription factor. ( Adapted from Reproductive Endocrinology by Yen and Jaffe 1991).



### 1.5.2 Mechanisms of Action of Steroid Receptors

Evans (1988) showed that the ER and PR are members of a subfamily of intracellular receptors (IRs) which include the receptors of androgen (AR), aldosterone (MR) and glucocorticoid (GR). The cellular concentration of the IRs is extremely low under normal physiological conditions, rarely exceeding 0.01% of the total cellular protein (McDonnel, Mangelsdorf, Pike et al. 1987). Currently, significant experimental evidence by Smith and Toft (1993) indicates that all the sex steroid receptors reside in the nucleus prior to ligand binding. They have also showed that the mechanism by which steroid receptors mediate their biological effects in target cells is similar. In the absence of hormone, the latent receptor resides in a large macromolecular complex comprising heat-shock proteins (hsp) 90, hsp70, p59 and other proteins. No clear function has yet been identified for the heat-shock proteins in sex steroid hormone action.

Sex steroids exert their effects on gene transcription via specific intracellular receptor proteins. Genetic and biochemical evidence suggests that signal transduction to the nucleus occurs in a series of distinct steps as outlined in the model below by Henderson, Philibert, Roy and Teutsch (1995) (Fig 3.2). In brief, steroids enter the cell passively where they encounter their cognate receptor in a complex with heat-shock proteins. The binding

ligand initiates a cascade of molecular events, including phosphorylation, dimerization, nuclear translocation, interaction with specific DNA response elements (SRE), and recruitment of adaptor proteins which allow the steroid receptor to productively interact with the general transcription apparatus (GTA). The transcriptional effects of sex steroids on RNA polymerase activity are determined ultimately by the cellular and promoter contexts of the IR bound to DNA.

A publication by Potgiarter et al (1995) concluded that a definite biochemical and physiological link exists between ER and PR. Progesterone receptor levels appear to be stimulated by estrogens, while the ER and PR levels are down-regulated by progesterone. The latter researchers have further speculated that the regulation of the physiological action of both estrogen and progesterone hormones is dependent on the relative concentrations of both receptors. Green (1990) had earlier demonstrated that the ER modulates gene expression by binding to specific estrogen responsive elements causing the activation of proto-oncogenes, autocrine and paracrine growth factors, as well as their receptors. From Green's demonstration Potgiarter et al (1995) postulated that any disturbance in the relative PR/ER could lead to either increased or decreased gene activation and expression of growth factors and/or their receptors, effecting aberrant growth of myometrial cells and as a result causing the formation of fibroids.





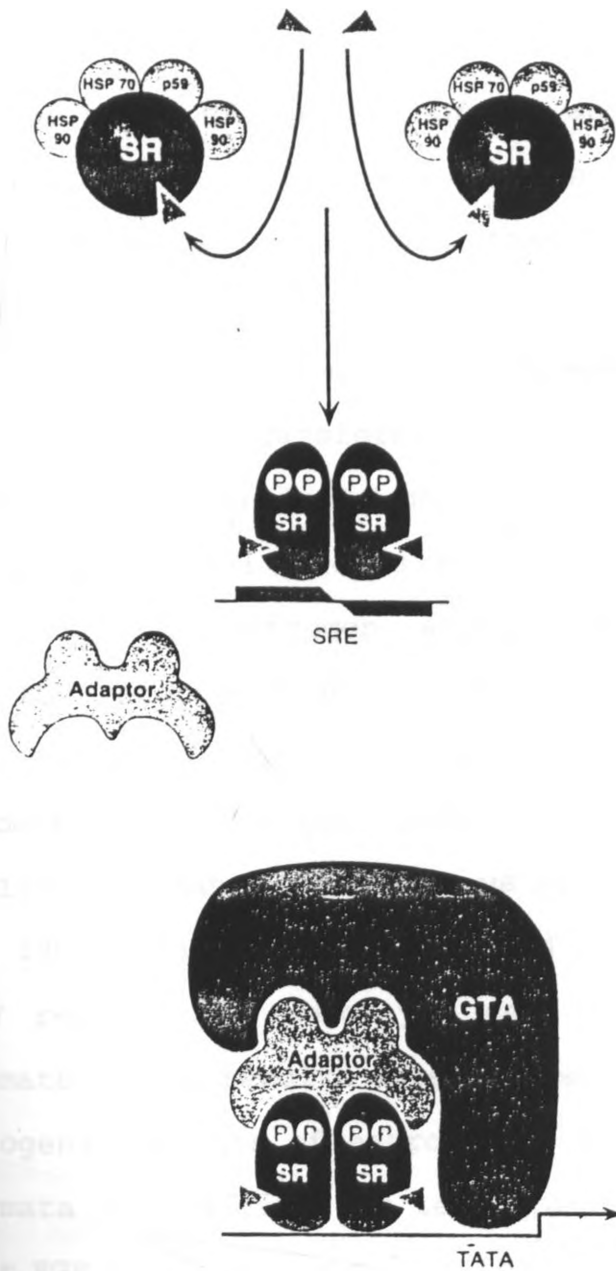
Fig. 3.2 The mechanism of action of sex steroid hormone receptors  
(Adapted from steroid receptors and antihormones edited  
by Henderson et al. 1995).

SR = Cognate receptor

GTA = General transcription apparatus.

SRE = Specific DNA response elements.

Fig. 3.2.



POSSIBLE STEPS

Delivery of Steroid to cell

Steroid binding

Conformational change

Phosphorylation

Displacement of heat shock proteins

Dimerization

Nuclear translocation

Interaction with DNA

Phosphorylation

Recruitment of adaptor protein

Interaction with GTA

Modulation of RNA polymerase activity

### 1.5.3 Possible Role of Growth Factors in Development of Leiomyomata

In the study of steroid hormone and receptors involvement in leiomyomata the role of growth factors cannot be neglected. There is increasing evidence to suggest that the growth-promoting effects of estrogen on the uterus are mediated by local production of peptide growth factors (Yeh, Rein and Nowak 1991). Epidermal growth factors (EGF) and transforming growth factor-alpha (TGF-a)- a structural and biochemical homolog of EGF- have been shown to have similar physiological effects on the uterus. In animal studies (e.g monkeys and cows), estrogen stimulates the expression of uterine EGF and EGF receptor messenger ribonucleic acid (mRNA). In these studies, EGF binding activity has been reported for both myometrium and leiomyomata (Yeh & Yeh, 1989; Huet-Hudson, Chakraborty, Suzuki et al. 1990; Hofmann, Rao, Barrows et al. 1984).

In 1991, Yeh et al, established the presence of mRNA for EGF and EGF receptor in monolayer cell cultures of myometrium and leiomyomata. These results were consistent with the hypothesis that the mitogenic effects of estrogen on human uterine myometrium and leiomyomata are mediated by local production of EGF. A potential role for EGF and its receptor in the regulation of human myometrial and leiomyomata growth is also consistent with the observations of Lumsden, West, Bamley, Rungay, and Baird (1988) who demonstrated

that shrinkage of uterine leiomyomata with GnRH-a therapy is associated with a reduction in the uterine EGF binding sites.

In a report by Dawood and Khan-Dawood (1994) wherein insulin-like growth factor I (IGF-I) gene expression in human leiomyomas was demonstrated and the complete nucleotide sequence of the messenger ribonucleic acid (mRNA) for IGF-I elucidated, serum IGF-I levels were found to be decreased in patients with leiomyomata while on treatment with GnRH-a, rendering them hypoestrogenic, but not with those on placebo. They also demonstrated that circulating levels of estrogen are not increased in patients with fibroids compared with normal patients. Hypoestrogenemia induced by GnRH-a therapy of women with uterine fibroid reduces plasma IGF-I levels as well as secretion of IGF-I by explants of such treated tumours . Therefore, it appears that normal levels of estrogen during the menstrual cycle may play a permissive role in the maintenance of uterine leiomyomas whereas hypogonadal levels will induce regression.

## 1.6 MANAGEMENT OF LEIOMYOMATA

Management modalities of fibroids have depended on the symptoms and reproductive status of the patient. The available modalities include no treatment, palliative treatment, polypectomy and vaginal myomectomy, abdominal myomectomy, and hysterectomy (Tindall, 1987).

No treatment: Small symptomless fibroids (smaller than 10 weeks pregnancy) discovered accidentally do not require treatment, although the patient should be kept under observation, in order to monitor the growth and possible complications of the fibroids.

Palliative treatment: If for any good reason operation has to be postponed, menorrhagia can sometimes be temporarily controlled by administering danazole or norethisterone acetate. Alternatively, an estrogen-progesterone preparation, such as is used for contraception purposes, can be given orally whilst awaiting admission for surgery.

Polypectomy and vaginal myomectomy: Tumours presenting at or through the vaginal cervix are removed vaginally, taking care to exclude associated uterine inversion. Their removal whole or piecemeal through the cervix, can be a difficult and traumatic procedure.

Abdominal myomectomy: Abdominal myomectomy is the operation of choice in most patients less than 40 years of age, and in older ones who desire their menstrual and reproductive functions. The disadvantage of myomectomy is that menorrhagia persists after operation in 1-5 % of cases, recurrence rate of fibroids after myomectomy is 5-10 percent.

Hysterectomy: This is the best treatment for uterine fibroids in women over the age of 40 years and in those not anxious for more children. The cervix as well as the corpus is removed in most cases but the ovaries, if normal should be conserved in premenopausal women.

## HORMONAL THERAPY IN THE MANAGEMENT OF FIBROIDS.

Research work done on steroid hormone and receptor involvement in the pathophysiology of fibroids has revolutionized the management of uterine leiomyomata. The search for alternate methods of management of fibroids has been prompted by the frequently reported postoperative complications, and the fact that some patients desire to be pregnant. Such an alternative method involves an endocrine approach that is currently being used for the treatment of various hormone-dependent tumours and endometriosis (Jenson, 1995). In this regard Goland et al (1989) in their work used D-Trp-6-LH-RH microcapsules, a long acting LH-RH agonist, for the treatment of 26 patients with uterine leiomyomata as an alternative approach to surgery. These patients exhibited a marked shrinkage of the uterus. In 1988, Ran, Beiber, Wood and Peping had also shown that GnRH-a (Leuprolide) administered for six months by either 50mg subcutaneously (sc) or monthly 3.75mg depot intramuscular (im) injection, the size of the leiomyomata regressed on average 42 per cent after 6 months of treatment.

Gonadotropin releasing hormone-agonist (GnRH-a) administration produces a biphasic endocrine response with an initial surge followed by a decline and sustained low level gonadotropin and general steroid secretion. The decrease in uterine size is dependent on the hypoestrogenic state which follow down regulation of the pituitary GnRH receptors and desensitization of gonatropes (Friedman et al. 1989). Consequently, it has been proposed to

treat leiomyomata by estrogen-progesterone diminution which can be achieved by GnRH-a (Buttaran et al. 1981). In 1986 Van Lemsden showed that treatment of women with fibroids with the GnRH-a results in an average decrease in uterine volume by 40 to 50 per cent.

Friedman et al (1989) in an experiment to evaluate the efficacy of leuprolide acetate depot in the treatment of uterine leiomyomata showed that treatment with leuprolide acetate depot caused a mean reduction of uterine size of 40 percent, and this volume decrease was comparable to that reported by earlier studies (Van Leusden 1986). Maximum reduction was achieved by 12 weeks of therapy, with no further change observed after 24 weeks of therapy. However, mean uterine volume increased to 88 percent of pre-treated size within 3 months of cessation of leuprolide therapy in 16 patients who completed 3 months of follow-up.

The regrowth of the uterine leiomyomata soon after termination of depot leuprolide injection suggest that primary medical therapy with a GnRH-a alone is unlikely to permanently eradicate symptoms due to leiomyomata. Rather, leuprolide may be useful as a preoperative adjuvant for hysterectomy and myomectomy. The decrease in uterine volume may make surgery technically possible with less blood loss in some patients. However, further studies are necessary to evaluate leuprolide as a preoperative adjuvant and assess its safety in long-term use.

A synthetic GnRH-a goserelin, called Zoladex, from Zenaca Pharmaceuticals has been shown to achieve an effective and

consistent reduction in serum estradiol concentrations through a direct action on the pituitary gland. The estradiol levels fall to postmenopausal levels within 3 weeks (Mettler, Steinmuller and Sunschmann 1991; Venturini, Fasce and Constantinnis 1990). Preoperative treatment with Zoladex has been shown to produce a 37 percent reduction in fibroid diameter after 6 months (Auber 1990). Additionally the use of Zoladex in conjunction with surgery may allow vaginal rather than abdominal hysterectomy to be undertaken because of the reduction in uterine size. Relief of symptoms with Zoladex treatment may enable surgery to be postponed in patients who are approaching the presumed age of menopause (Auber 1990).

Though the prevailing opinion that the reduction in uterine and fibroid volume associated with chronic administration of GnRH-a is believed to be secondary to resultant hypoestrogenic state (Friedman et al. 1989) the mechanisms mediating the decrease in uterine and fibroid volume in GnRH-a treated patients are poorly defined. Hence research has moved forward to determine the concentration of steroid hormone receptors in leiomyomata in a bid to define properly the etiology, pathophysiology and therapy of the disease.

Findings reported from work done to determine the concentration of these receptors in this disease is at variance and this study seeks to address in part this controversy. Recent publications (Potgieter et al. 1995) in trying to resolve this controversy showed that these receptor levels were higher in the fibroid than in the normal myometrium. However in the above study



the fibroid specimen and the normal myometrial sample were from different patients i.e control and experimental tissues were from different subjects. In the present study attempt was made to overcome this problem by using diseased and normal tissues from the same patients.

#### 1.7 JUSTIFICATION FOR THE STUDY

Uterine leiomyomata is a common problem in women of reproductive age. It is associated with infertility, poor reproductive performance and gynaecological morbidity. The black population has been reported to have a higher incidence of leiomyomata and no apparent cause of this racial association has been identified (Tindall, 1987). The high incidence of this disease has been reported in black women living among Caucasian populations. The need for intensive research regarding leiomyomas in the black population cannot be over emphasized. Infertility in this population is viewed as a social stigma since childbearing is regarded as central in matrimonial harmony and child adoption is yet to be appreciated. The incidence of surgical complications are higher in the developing countries than in the developed countries, hence surgery which at present is the main method of management of fibroids will result in more side effects in the black population.

Presently, research to identify the etiology and pathogenesis

of the disease is centered around steroid hormones and steroid hormone receptors. The use of GnRH-a in the treatment of this disease is limited to reducing symptoms and the reduction of uterine size to facilitate operation. The management and prevention of this disease has been complicated by the fact that the pathogenesis is enigmatic. Scientific work has generated a consensus on the involvement of steroid hormones and steroid hormone receptors in uterine fibroids but there is controversy as to the levels of these hormones and receptors.

The aim of this study is therefore to try to resolve some of the controversy about the quantitative levels of steroid hormones ( $E_2$  &  $P_4$ ) and steroid hormone receptors (ER & PR) in leiomyomas and the normal myometrium and to determine these levels in the black population in Kenya so that these attributes of fibroids can be exploited in the clinical management of uterine leiomyomas.

## 1.8 OBJECTIVES OF THE STUDY

### **Broad Objective:**

The broad objective was to determine the levels of estradiol, progesterone, estrogen and progesterone receptor concentrations in the uterine leiomyomata.

### **Specific Objectives:**

The specific objectives were to measure and compare estradiol

and progesterone levels in the fibroid tissue and the adjacent normal myometrium in the same patient, and to measure and compare the concentration of estrogen and progesterone receptors in the fibroid tissue and the adjacent normal myometrium in the same patient. These levels were also compared in the follicular and luteal phases of the menstrual cycle.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 STUDY DESIGN

This was a descriptive and comparative study; describing the levels of estradiol ( $E_2$ ), progesterone ( $P_4$ ), estrogen receptors (ER) and progesterone receptors (PR) in the leiomyomata and the adjacent normal myometria; and comparing these levels in the leiomyomata against those in the adjacent myometria.

#### 2.2 STUDY AREA

This study was carried out in the Department of Obstetrics and Gynaecology and the Department of Human Pathology in the Kenyatta National Hospital Nairobi; and the Reproductive Biology Unit in the College of Biological and Physical Sciences, University of Nairobi - Kenya.

### 2.2.1 Kenyatta National Hospital

Kenyatta National Hospital (KNH) is the national referral and teaching hospital of the Republic of Kenya. It has a 3,000 bed capacity and receives referrals from all the Provinces of the country and neighbouring countries for specialized care. It also serves a population of the city of Nairobi and environs which has approximately 2 million inhabitants.

Kenyatta National Hospital serves as the teaching hospital for the college of Health Sciences, University of Nairobi, training medical professionals at under-graduate and post-graduate levels. It also trains para-medical staff in liaison with the college of Health Professionals (that is, Medical Training Centre). The hospital comprises of various departments, Obstetrics and Gynaecology being one of them.

#### The Department of Obstetrics and Gynaecology.

This department provides both inpatient and outpatient obstetrics and gynaecologic services. The out-patient services are provided at the Casualty Department, Antenatal, Postnatal and Gynaecology Consultant Clinics, a Family Planning Clinic and facility which provides diagnostic laparoscopic and surgical sterilization services.

In-patient services occupy the ground and first floors of the tower complex and comprises of a labour ward, six lying-in wards and a newborn unit. Ward 44 is located in the Old Hospital Wing for Gynaecology Oncology Cases.

### The Gynaecology Unit:

This comprises of an Out-patient Consultation Clinic, Wards 4, 5 and 6 on the first floor of the tower block and Oncology Ward 44 in the Old Hospital Wing. Each of the wards has a 32 bed capacity.

### The Gynaecology Clinic:

This is located in Consultant Clinic No.18 and is held on Tuesday, Wednesday and Thursday afternoons. Monday is reserved for special infertility and adolescent antenatal clinic. Each firm has one clinic day per week. The commonest cases seen are those of infertility, accounting to 2/3 of the patients, uterine fibroids and abnormal uterine bleeding. Patients are fully investigated before admission for an operative procedure in ward 4 and 5 to shorten Hospital stay and hence improve utilization.

### Admission:

Emergency gynaecological admission - Ward 6 - are made usually through the Casualty Department.

Non-Emergency admissions - Ward 4 and 5 - are made through the out-patient consultation. The available beds are divided among the three firms for non-emergency surgery; for example vesico-vaginal fistulae repairs, hysterectomy and tuboplasty.

### Gynaecology Operations:

Gynaecology operations are done in the hospital main theater on Mondays and Fridays. Pre-operatively, haemograms, urea and electrolytes, HIV screening and group and cross matching are done in addition to specific investigations dictated by the pathology of

each case e.g fibroids - ultrasound and pap smears.

Routine theater lists for "cold" wards are prepared on the firm basis. Most of the major operations such as vesico-vaginal fistula repair (V.V.F), total abdominal hysterectomy (T.A.H), radical vulvectomy etc are carried out in the routine theater lists.

### **2.2.2 Department of Human Pathology**

The Department of Human Pathology is one of the departments in the Kenyatta National Hospital - Nairobi. It offers training to Medical (under-graduate and post-graduate) and Para-medical students. It also offers service to other departments of the hospital and faculties of the College of Health Sciences in the areas of Surgical pathology (Histopathology), Cytology, Morbid anatomy (Autopsies for KNH) and Immunology.

Research in this department is being carried out by staff members and post-graduate students. Research areas include cervical cancer, schistosomiasis, HIV/AIDS and placenta pathology in HIV. Support for teaching, research and staff training has been from CIDA and Belgium in the recent past.

### **2.2.3 Reproductive Biology Unit**

The Reproductive Biology Unit (RBU) is an informal research group of scientists at Chiromo Campus, College of Biological and Physical Sciences, University of Nairobi, who are involved in basic researches in mammalian reproduction so as to gain better

understanding of the reproductive processes on which better means of regulation of fertility can be based.

The Unit i.e RBU evolved from research of individual scientists but has been consolidated into a functional, cohesive unit, on provision of institutional strengthening funds by WHO-HRP and other donors like IAEA, IDRC. NCST and the University of Nairobi itself.

The unit comprises a core group of scientists and technicians from the Departments of Animal Physiology, Veterinary Anatomy, Biochemistry, Zoology, Reproduction and Obstetrics and the Kenya Medical Research Institute. It carries out physiological, biochemical, and anatomical research in domestic, laboratory and wild animals which may provide suitable models for human and veterinary reproduction in the areas of:

- (a) Sperm maturation and epididymal physiology;
- (b) Implantation and early pregnancy;
- (c) Infertility problems caused by parasitic diseases and/or environmental factors;and
- (d) Methodological developments for endocrine investigations and managements.

Techniques for RIA, RRA, EIA and chemiluminescence have been developed, as has in vitro culture system for corpus luteum and Leydig cells.



## 2.3 SELECTION OF PATIENTS FOR THE STUDY

This study was conducted among women undergoing elective hysterectomy for uterine fibroids at the Kenyatta National Hospital (Obstetrical and Gynaecological ward and theater) Nairobi during the period August to December 1995. The Research Project Proposal for this study had been dully submitted and approved by the ethical committee in the same hospital. In this hospital gynaecological cases for elective surgery are usually operated upon on Mondays and Thursdays. These patients are admitted into the ward at least 24 hours prior to the day of operation.

From the list of booked patients, those diagnosed with uterine fibroids and scheduled for hysterectomy were identified. On average they were usually three of such cases each week. Hospital files and physical examinations were used to assess the medical status of these patients and the findings recorded in a proforma form (Appendix 1). Socio-demographic and reproductive data included: name, age, date of operation, last menstrual period, menstrual patterns in the last 3 months, parity, drug treatment and uterine size.

### 2.3.1 Characteristics of Patients Selected

Cases used in this study were selected among women diagnosed for uterine fibroid and admitted for hysterectomy. Only women of reproductive age (20-45 years) were included in the study. Women

with fibroids co-existing with cancers diagnosed before or during surgery were excluded. Also excluded were women on hormonal therapy e.g patients on estrogen and progesterone.

## **2.4 SAMPLE SIZE DETERMINATION**

The sample size of twenty (Appendix II) was attained by recruiting all women who fulfilled the inclusion criteria as above. This took from August to December 1995.

## **2.5 MATERIALS**

### **2.5.1 Tissue Samples**

Specimens of uterine leiomyomata and the adjacent normal myometria were collected from twenty patients undergoing hysterectomy in the gynaecology theater. While in the theater, as soon as the uterus was removed the fibroid tissues was separated from the adjacent normal myometrium by trimming with a scalpel blade and forceps.

Paired specimens (fibroid and myometrium) weighing approximately 20-30 g were collected and immediately transferred, frozen into ice block containers, and transported to the laboratory

where multiple tissue samples were removed for histological examination and the rest frozen at -20°C and later processed for the following:

- (a) Reproductive hormones i.e estradiol and progesterone and
- (b) Receptor contents i.e estrogen and progesterone receptors.

### 2.5.2 Solvents and Reagents

The list and source of chemicals used in these studies is given below:

Dithiothreitol, Triton x, Bovine serum albumin (BSA), Dextran activated charcoal, Diestrine and Formasaline were obtained from Sigma. Antibody (Antiserum to estradiol-17B), (2,4,6,7-3H) estradiol and 17 $\alpha$ -Hydroxyl(1,2,6,7-3H) Progesterone were from the WHO RIA Reagent Program. Sodium azide, Sodium hydroxide, Sodium carbonate, gelatin and Follin Ciocaltea reagent were obtained from Merck.

Glycerol, Sodium/Potassium tartrate, Copper sulphate, Xylene, Haematoxylin and Eosin, TEDG-buffer (10mM Tris-1.5mM EDTA-1mM dithiothreitol containing 10% glycerol at pH 7.4) and Protein buffer saline (PBS) were from BDH Chemicals. Toluene and Diethyl ether from M&B. Paraffin wax from Aldrich. PPO (2,5-Diphenyloxazole) from Du Pont.

## 2.6 PREPARATION OF CYTOSOL

The method for preparation of cytosol followed the procedures outlined by Bauer and Gorell (1980) for ovine uterus. The tissues (myometria and leiomyomata) were removed from the ice block containers and allowed to thaw. Thereafter, 10 g of each tissue was weighed out then cut into small pieces using sharp scalpel blades. These pieces were later transferred into an electric homogenizer and 40 ml of TEDG buffer (consisting of 10mM Tris-1.5mM EDTA-1mM dithiothreitol containing 10% glycerol at pH 7.4) was added. The mixture was then thoroughly homogenized for 18 minutes with 15 bursts.

The homogenization was accomplished at 0-4°C with 10 second bursts using a pre-chilled Sanyo SM 3050 tissue blender followed by 1 minute cooling intervals. The homogenate was centrifuged in a Sorvall RC-5B Centrifuged at 27,000 x g for 40 minutes to yield a supernatant (referred to as cytosol in the remainder of this study). The cytosolic fluids (myometria and leiomyomata) were tipped into Lp3 tubes and stored in a freezer until extraction time for the measurement of reproductive hormone levels and receptor contents.

## 2.7 DETERMINATION OF ESTROGEN AND PROGESTERONE RECEPTORS

### 2.7.1 Pilot study

A pilot study was first carried out with various concentrations of labelled  $E_2$  antigen and doubling dilutions of cytosols to determine the right concentrations of each to obtain optimum levels for which a comparative study could be done. Similar studies were also carried out with various levels of labelled  $P_4$ . The results used to determine the optimum working dilutions of cytosol and optimum tracer concentrations for myometria and leiomyomata are shown in Chapter 3.

### 2.7.2 Determination of Binding Sites

The cytosolic fluids (myometria and leiomyomata) that had been prepared and stored in the freezer ( $-20^{\circ}C$ ) were removed and thawed. Dilutions of 1:4 (in TEDG buffer) of these fluids were prepared and 200ul of each pipetted in triplicates into Lp3 tubes. Meanwhile tracer [(2,4,6,7- $^3H$ )Estradiol] solutions were prepared by taking 150ul of the stock solution into a tube and the solvent evaporated in a vacuum drier. The pellet redissolved in 15ml of EDTA-buffer (EDTA, 3 mmol/l; Tris-HCL, 20 mmol/l; dithreitol, 1 mmol/l; 0.01% $NaN_3$ ; 0.01%BSA; 10% glycerol; pH 7.8) and allowed to stand for 30 minutes. This solution then contains 3.7 k Bq/ml (100nCi/ml) of which 0.1 ml (10nCi) was added to the Lp3 tubes already containing the cytosol and vortex mixed for 1 minute. The mixture was then

incubated at 4°C for 48 hours. Using centrifugation (1000 x g for 10 minutes at 4°C) bound and unbound ligand were then separated by charcoal adsorption ( addition of 300ul EDTA buffer containing 1% activated charcoal and 0.025% dextran 60). Following centrifugation the supernatant was removed for counting in a Beckman Ls-7000 Liquid Scintillation Counter. The scintillation fluid consisted of 7.5g PPO (2,5-Diphenylloxazole) per litre of toluene.

The same procedure was repeated for 3HP<sub>4</sub> (17 $\alpha$ -Hydroxyl[1,2,6,7-<sup>3</sup>H]Progesterone).

### 2.7.3 Determination of Cytosolic Protein

The method for the determination of protein concentration in the cytosol followed the Lowry et al.(1951) method adapted and validated by the Department of Biochemistry University of Nairobi outlined below:

0.5ml of samples (cytosol) and concentrations (0.002 - 0.01g per 10ml) of Bovine Serum Albumin (Fraction 5 Lot 902787 B grade) were first incubated with 2.3ml of mixture A containing 0.1mM sodium hydroxide, 0.2M sodium carbonate, 0.7mM sodium/potassium tartrate and 20mM copper sulphate for 10 minutes at room temperature. 0.22ml of Follin Ciocalteu reagent was then added to the reaction mixture and further incubated at room temperature for 30 minutes.

The amount of colour developed was then measured by reading the absorbance at 750nm wavelength with a Perkin-Elmer 550S Spectrophotometer. A standard curve of optical density (OD) versus protein (BSA) concentration was obtained and the protein levels in each sample read from the curve and the amount finally expressed in micrograms.

## 2.8

### STATISTICAL PROCEDURES

#### Method of Calculation of the Bound Labelled Hormone on both Estrogen and Progesterone Receptor Proteins.

The method used to calculate the estrogen and progesterone receptors in the myometria and leiomyomata was done indirectly by determining the amount of labelled hormone bound unto the receptor protein through the steps outlined below:

#### Step I. Calculation of percentage bindings of labelled hormones.

The percentage bindings of the labelled hormone in each cytosol (myometrium and leiomyomata) was calculated (after incubation, charcoal separation and counting) using the formula below;

$$(MC - NSB) / TC \times 100$$

Where: MC = mean count => fraction of radiolabelled hormone

bound to the receptor site.

: TC = total count => amount of radiolabelled hormone put into each tube.

: NSB = non-specific binding.

=> the net mean count = the fraction of the radiolabelled hormone bound to the receptor site less non-specific binding.

Step II Calculation of the amount of bound antigen per ml of cytosolic fluid

From the method of determination of binding sites described above, fixed amounts (Y) of radiolabelled hormone (0.124 and 0.105 umoles of estradiol and progesterone respectively; calculations based on figures obtained from WHO Matched Reagent Programme Manual 1993 and molecular weights from Steroids (1985) in 100 ul volumes (TC) were added to 200 ul cytosol.

Therefore, amount of radiolabelled hormone bound in the 200 ul cytosolic fluid is given by:

$$(X/TC \times 100)Y \text{ in fmoles.}$$

Where: X = average counts obtained after charcoal separation.

: TC = total count

: Y = amount of antigen contained in the mean total count.



Therefore 1ml of cytosol will contain:

$$(X/TC \times 100)Y \times 1/0.2 \text{ fmol/ml}$$

$$\Rightarrow (500X/TC)Y \text{ fmol/ml.}$$

Step III Calculation of bound hormone per mg cytosolic protein.

The amount of protein in the cytosol was determined by the Lowry method described earlier.

Assuming that 1ml of cytosol contains Q mg of protein:

$\Rightarrow (500X/TC)Y$  fmol of antigen bound to Q mg cytosol protein per ml.

therefore 1mg cytosol protein will bind

$$(500X / TC)Y \times 1/Q \text{ fmol/mg protein.}$$

Using the above calculations described in the steps above the amount of antigen ( [2,4,6,7-3H]Estradiol and 17 $\alpha$ -Hydroxyl [1,2,6,7-3H]Progesterone) bound to receptor protein (myometrium and leiomyomata) was determined.

These methods were used to indirectly estimate the amount of estrogen and progesterone receptors in the myometria and leiomyomata of the twenty patients studied. The means of these values and standard mean errors were calculated and results shown on Data analysis and Dummy Tables in chapter three.

## 2.9 HORMONAL ANALYSIS IN THE LEIOMYOMATA AND MYOMETRIA

The estradiol and progesterone levels in the samples were determined by radioimmunoassay (RIA) using method outlined in the WHO Matched Reagent Programme Method Manual by Sufi et al. (1993).

Briefly 500ul and 200 ul of cytosol was extracted with 10 times volume of diethyl ether for estradiol and progesterone respectively using extraction tubes. The mixture was separated by freezing the aqueous and tipping off the ether into pre-labelled clean Lp3 tubes and then dried down by transferring the tubes containing the extracts to a warm water bath. In each tube 2ml of steroid assay buffer (Phosphate buffered saline) was added. This was then reconstituted with 2ml PBS containing gelatin and sodium azide at pH 7.4. To 0.5ml of this 100ul (10,000 counts per minute) of tracer and 100ul of antibody was added. The mixture were then incubated overnight at 4°C and the bound antigens separated from the free using pre-chilled dextran activated charcoal and centrifuged at 1000 x g for 10 minutes. The bound hormone was tipped off into clean vials and the unbound disposed. The bound hormone was then scintillated with 4 ml toluene/PPO mixture and counted as earlier described.

From these results the amount of estradiol and progesterone in the myometria and leiomyomata were then calculated and mean values and standard mean error shown in Chapter 3.

### Hormonal assay validation:

The intra-assay coefficient of variation (CV) for E<sub>2</sub> was 2% at 184pm/l and 4% at 1.56nm/l for P<sub>4</sub>. The inter-assay coefficient of variation was 7% for both E<sub>2</sub> and P<sub>4</sub>. The assay sensitivity (minimum detectable dose) was 97% at two standard deviation (SD) for E<sub>2</sub> and 96% at one SD for P<sub>4</sub>.

### 2.10 LEVEL OF STATISTICAL SIGNIFICANCE

The statistical analysis for the various levels of receptors and hormones in this study were performed using the " Student t-test" proposed by Gosset in 1908 (Norman, Schrot, Balch et al. 1970).

With the "Student t- test" formula the null hypothesis assumption is tested whereby a probability is used as guide in deciding whether the two sample results are really different. A P-value of 0.05 or less was taken as showing significant difference, (formula and calculations in Appendix III and IV).

## 2.11 HISTOLOGY OF MYOMETRIAL AND FIBROID TISSUES.

This followed the method routinely used by the department of Human Pathology, Kenyatta National Hospital - Nairobi. This process involves the following:

- (a) Fixation of the tissues with 10% formol saline.
- (b) Trimming of the tissues on a wooden platter to 10x10 mm thickness.
- (c) Processing the tissues using a Citadel 1000 automatic tissue processor.
- (d) Embedding the tissue with paraffin wax in metal bases and allowing to solidify in tissue teks.
- (e) Sectioning the tissues with a microtome to 5um thickness. The blocked tissues were further cooled on ice then trimmed to expose and smoothen their surfaces. These were then sectioned on the microtome. The sections were first floated in 15-20% alcohol then in a warm water bath before being picked on slide glasses. The slides were then packed in a slide-rack and transferred into a hot air oven (60°C) to melt off the wax.
- (f) Staining done by routine Haematoxylin and Eosin stain.
- (g) Mounting of the sections:- one drop of d.p.x (diestrene plus plasticizer and xylene) put on the slides then covered with cover slip for microscopic examination.

## CHAPTER THREE

### RESULTS

#### 3.0 STATUS OF PATIENTS USED IN THE STUDY

Based on the method of data collection and sample size as described in the section for methods and materials, twenty patients were selected for investigation in this study. All the patients were pre-menopausal, black women in the age group 31-42 years. They were all scheduled for hysterectomy for confirmed leiomyomata. Of these patients 65% (13) suffered from secondary infertility while the rest 35% (7) had primary infertility.

Although all the patients had confirmed leiomyomata and associated infertilities their indications for surgery ranged from menorrhagia (prolonged menstrual bleeding), metrorrhagia (bleeding between periods) and occasional dysmenorrhoea (painful periods). The menstrual status of these patients determined by medical histories (as per last menstrual periods) suggested that 75% (15) had menstrual irregularities while 15% (3) were in the follicular and 10% (2) were in the luteal phase. It was observed that patients in the younger age group i.e below 31 years with fibroids and

associated infertilities preferred conservative management or myomectomy. The Socio-demographic characteristics and history of the twenty patients studied are summarized in Table 3.

**Table 1. Socio-demographic Characteristics and Reproductive History of the 20 patients**

<b>Parameter</b>	<b>Number of patients</b>	<b>Percentage</b>
<b>1. Age (years)</b>		
30-35	7	35
36-40	11	55
41-45	3	15
mean age 37		
<b>2. Parity</b>		
0	7	35
1	6	30
2	5	25
3	2	10
<b>3. Menstrual Patterns</b>		
Normal	5	25
(Follicular)	(3)	(15)
(Luteal)	(2)	(10)
Irregular	15	75
<b>4. Fertility History</b>		
Primary Infertility	7	35
Secondary Infertility	13	65
Normal Fertility	0	0
<b>5. Indication for Surgery</b>		
Menorrhagia	9	45
Menorrhagia & Metrorrhagia	3	15
Menorrhagia and Dysmenorrhoea	6	30
Menorrhagia, Metrorrhagia and dysmenorrhoea	2	10
<b>6. Uterine size</b>		
Range 12 - 28 weeks	20	100

### 3.1 RESULTS OF THE PILOT STUDY CARRIED OUT IN CHAPTER TWO

The pilot study was done to determine the optimum working dilutions of cytosol and the optimum tracer concentrations for myometria and leiomyomata.

The various cytosolic dilutions and the amount of tracer bound to receptors in the myometrium and fibroid, and the amount of tracer bound to the receptors at different tracer concentrations are shown in Appendix V. The results were represented in Figure 4 and 5. From Figure 4 it was observed that A to B represented the useful range i.e any reading between A and B can be assumed correct for both myometrium and fibroid. Point C represents the optimum working dilution of value 1:4. From Figure 5 it was observed that the optimum tracer concentration lies between the 3,500 count per minute (cpm) and 15,000 cpm, with approximate optimum of 10,000 cpm and 1:4 dilutions. A similar trend was observed when studies were carried out with labelled  $P_4$  antigen.

These optimum dilutions of 1:4 for cytosol and optimum values of 10,000 cpm for tracer concentrations obtained from the pilot study were subsequently used to determine the binding sites of tracer to cytosolic protein in the myometria and leiomyomata.



Figure 4: Cytosol dilutions versus percentage of tracer bound to receptors in the myometria and fibroids. A (0.2) to B (0.9) represent the working dilutions.

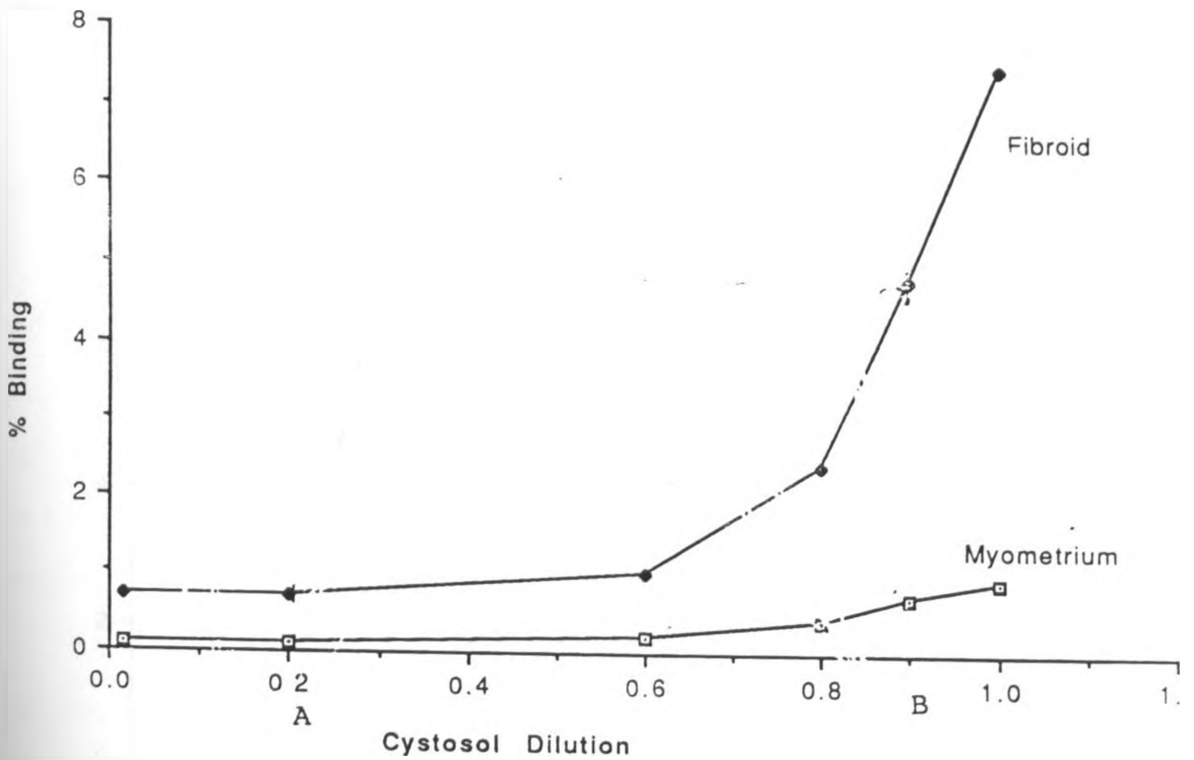
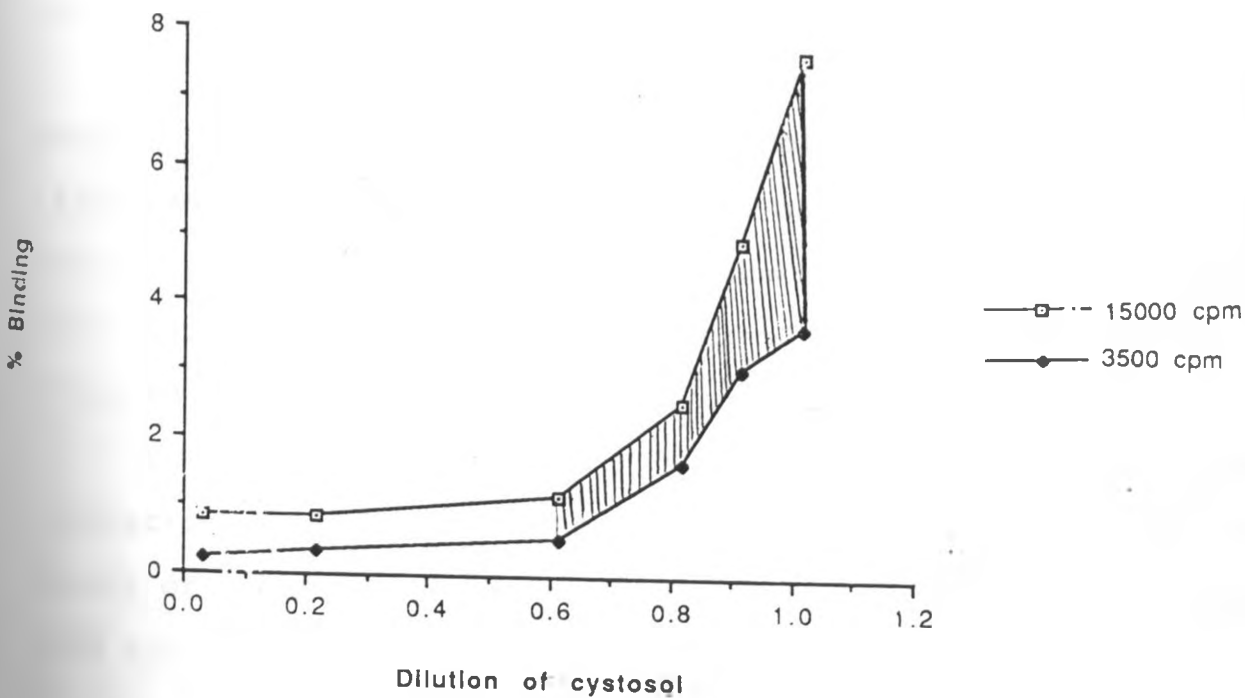


Figure 5. Cytosol dilutions versus percentage tracer bound to fibroid receptors at different tracer concentrations. Shaded area represent the best cytosol dilutions and tracer concentrations.



### 3.3 RESULTS OF ESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATION IN LEIOMYOMATA AND ADJACENT NORMAL MYOMETRIA

The results of estrogen and progesterone receptors (see Appendix VI) measured in the fibroid and adjacent normal myometrium of the twenty patients investigated in the study are represented in Figures 6 (ER), 7 (PR) and 8 (mean  $\pm$ SEM ER & PR).

The range of ER in the myometria was 16.3 - 22.5 fmol/mg of protein and the mean  $\pm$ SEM was 19.1  $\pm$ 0.4 fmol/mg of protein; whereas in the leiomyomata the range was 18.1 - 38.3 and mean  $\pm$ SEM 28.2  $\pm$ 1.6 fmol/mg of protein. Hence the uterine fibroid contained significantly higher levels of ER (147.6% :  $P < 0.001$ ) compared to the adjacent normal myometrium.

The range of PR in the myometria was 7.5 - 11.3 fmol/mg of protein and mean  $\pm$ SEM was 9.4  $\pm$ 0.2 fmol /mg of protein. In the leiomyomata the range was 11.9 - 21.9 and mean  $\pm$ SEM of 16.8  $\pm$ 0.7 fmol/mg of protein. These results show that the Uterine fibroid also contained significantly higher levels of PR (178.8% :  $P < 0.001$ ) compared to the adjacent normal myometrium.

It was also observed that the total ER in the uterine tissues (myometrium and fibroid) was approximately double (180.5%) the amount of total PR in the same tissues. The ER and PR values (Mean  $\pm$ SEM for fibroids and the myometrium are compared in Table 2

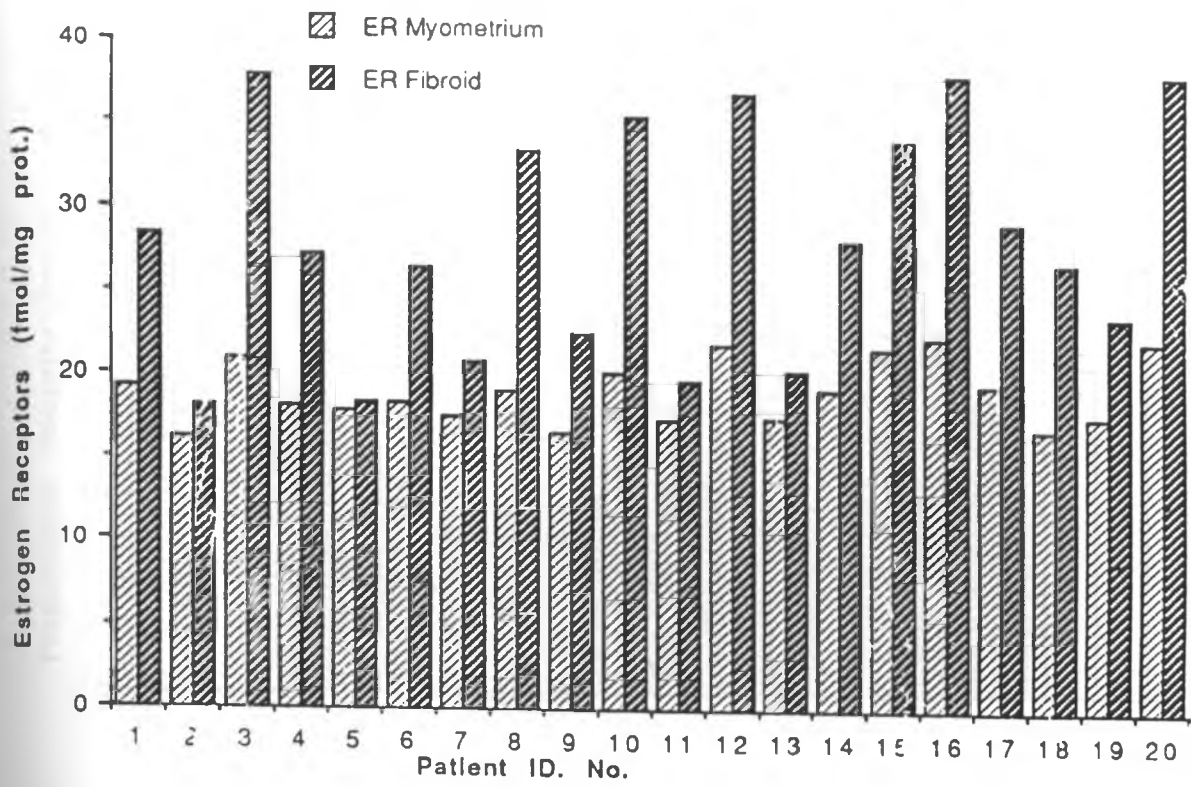


FIGURE 6: ER concentrations in fmol/mg protein in the myometrium and fibroid in the 20 patients investigated.

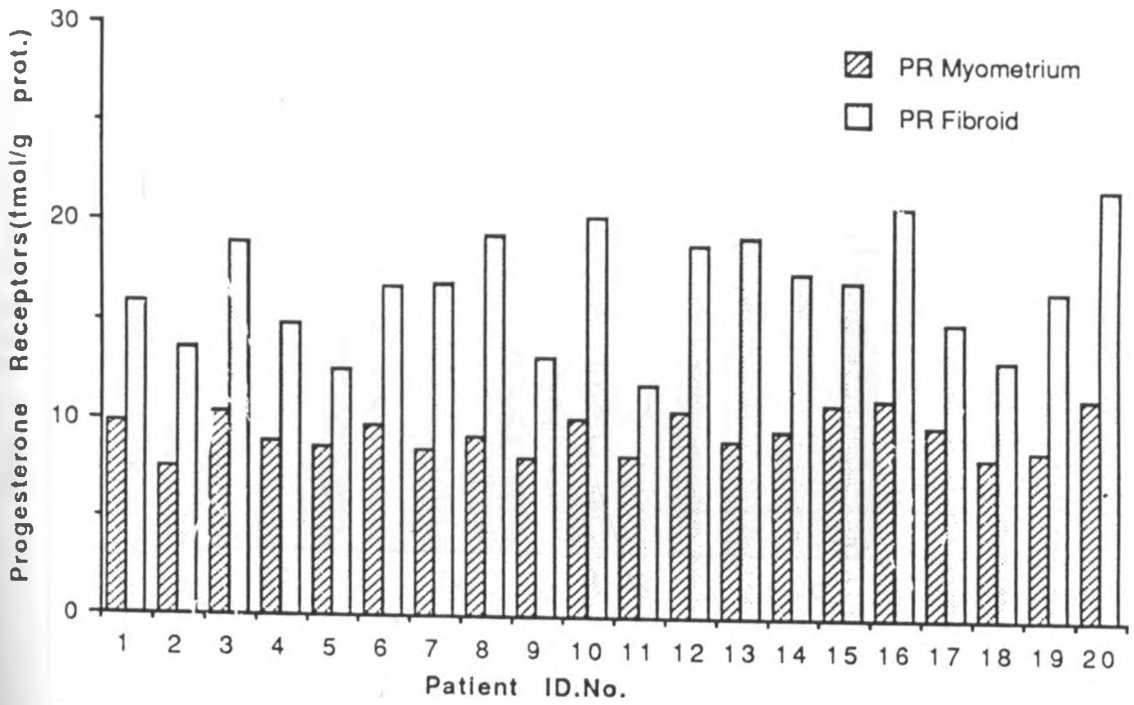


FIGURE 7: PR concentrations in fmol/mg protein in the myometrium and fibroid of 20 patients.

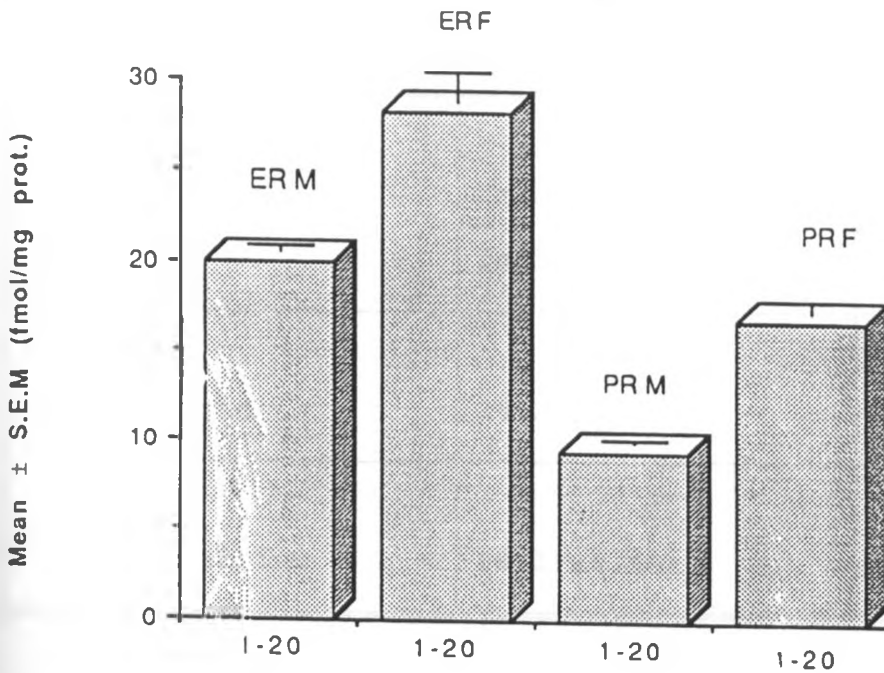


FIGURE 8: Mean  $\pm$ SEM. of ER and PR in the myometria and fibroids in fmol/gm protein. (n=20).

ERM = Estrogen receptor concentration in the myometria.

ERF = Estrogen receptor concentration in the fibroids.

PRM = Progesterone receptor concentration in the myometria.

PRF = Progesterone receptor concentration in the fibroids.

Table 2: Comparison of the mean  $\pm$ SEM of ER and PR in the fibroids and myometria. (prot. = protein)

n=20	ER (mean $\pm$ SEM) fmol/mg prot.	PR (mean $\pm$ SEM) fmol/mg prot.
Fibroid	28.2 $\pm$ 1.6	16.8 $\pm$ 0.7
Myometrium	19.1 $\pm$ 0.4	9.4 $\pm$ 0.2

### 3.4.            **ESTRADIOL AND PROGESTERONE LEVELS IN THE LEIOMYOMATA AND MYOMETRIA**

The range of  $E_2$  levels in the myometria was 972.5 - 1307.8 pmol/g tissue and the mean  $\pm$ SEM was 1117.6  $\pm$ 20.9 pmol/g tissue; whereas in the leiomyomata the range was 479.3 - 789.3 and the mean  $\pm$ SEM was 616.9  $\pm$ 19.8 pmol/g tissue.

The range of  $P_4$  levels in the myometria was 5.9 - 9.1 nmol/g tissue and mean  $\pm$ SEM 7.7  $\pm$ 0.25 nmol/g tissue. In the leiomyomata the range was 1.2 -5.1 and mean  $\pm$ SEM was 3.2  $\pm$ 0.34 nmol/g tissue.

These results show that the normal myometria contained significantly higher levels of estradiol (181.1% :  $P < 0.001$ ) and progesterone (240.6% :  $P < 0.001$ ) compared to the uterine leiomyomata (100%). It was also observed that the total progesterone level in the uterine tissues (fibroid and myometrium) was about six times higher (628.4%) than the total estradiol in the same tissues.

The above results are represented in Figures 9, 10 and 11. The comparative mean  $\pm$ SEM of  $E_2$  and  $P_4$  are shown in Table 3. (Estradiol and Progesterone levels in the fibroid and myometrium are shown in Appendix VII).



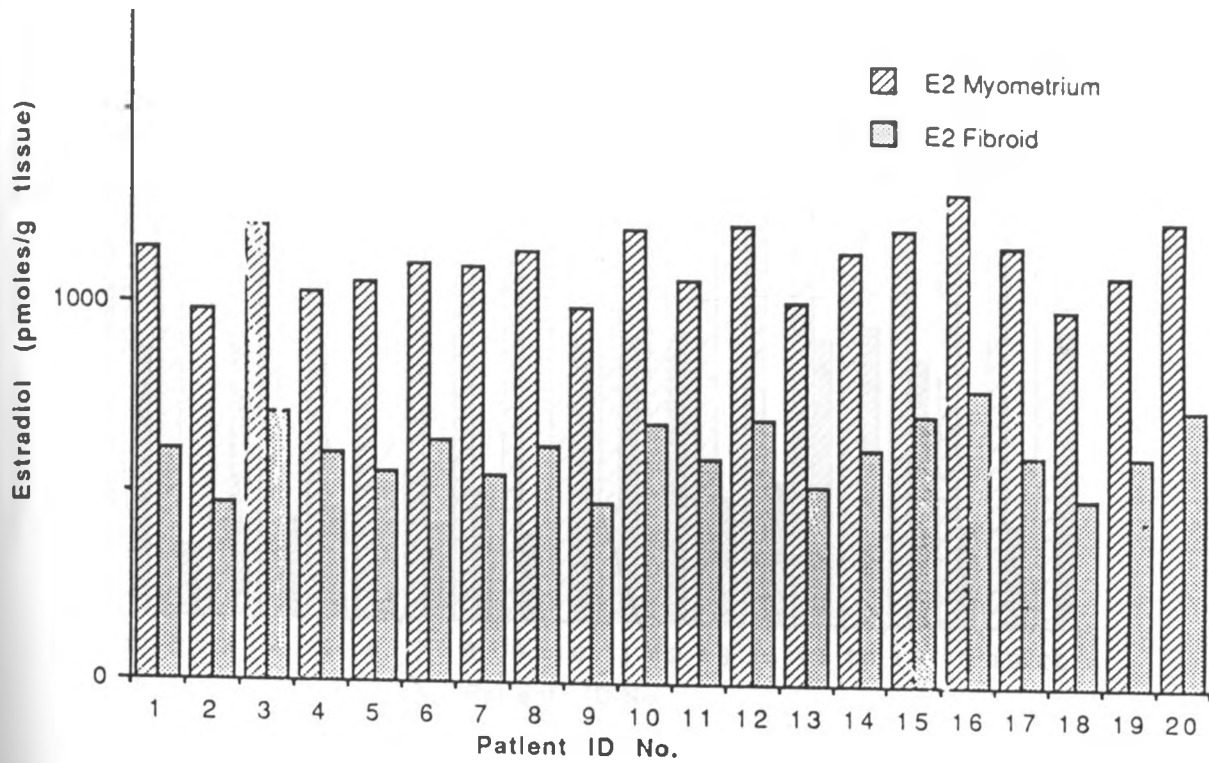


FIGURE 9: Estradiol levels in pmol/mg tissue in the myometria and fibroids.

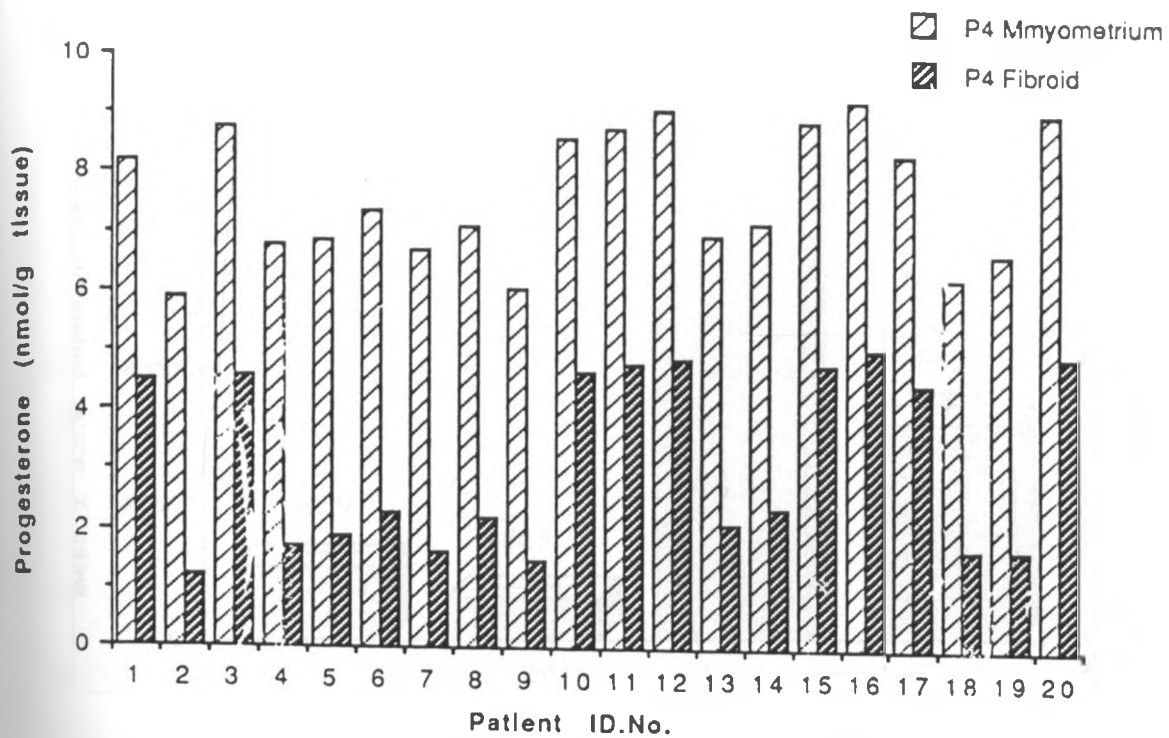


FIGURE 10: Progesterone levels in nmol/mg tissue in the myometria and fibroids.

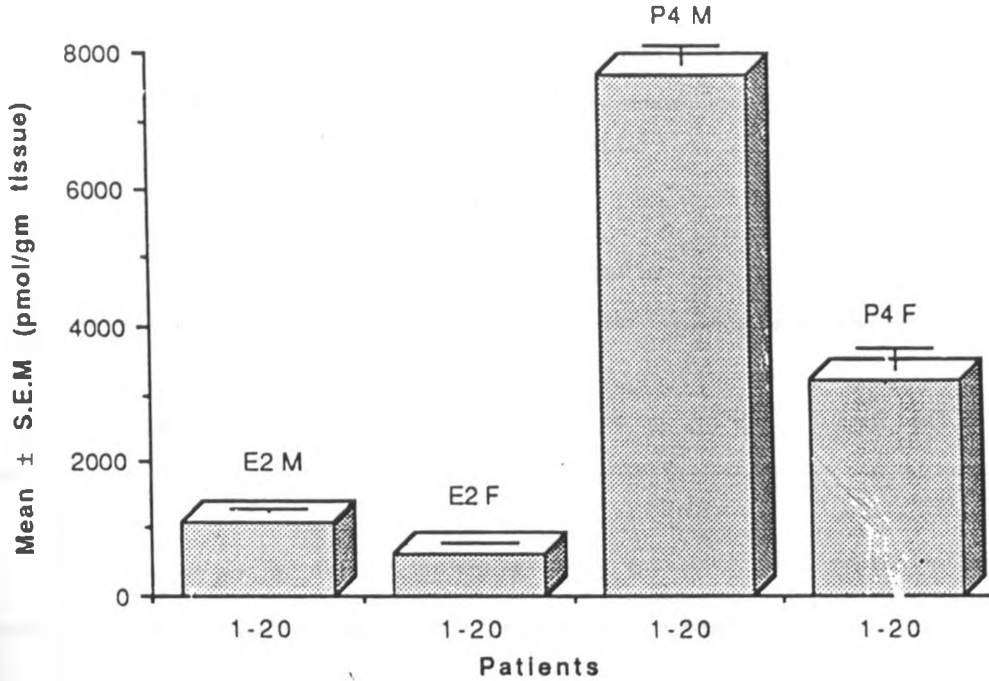


FIGURE 11: Mean  $\pm$ SEM of  $E_2$  and P, in the myometria and fibroids in pmol/mg tissue. (n=20).

- $E_2M$  = Estradiol concentration in the myometria.
- $E_2F$  = Estradiol concentration in the fibroids.
- $P_4M$  = Progesterone concentration in the myometria.
- $P_4F$  = Progesterone concentration in the fibroids.

Table 3: Comparison of the Mean  $\pm$ S.E.M. of  $E_2$  and  $P_4$  in the fibroids compared to the myometria.

n=20	$E_2$ (estradiol) mean $\pm$ SEM pmol/g tissue	$P_4$ (progesterone) mean $\pm$ SEM nmol/g tissue
Fibroid	616.9 $\pm$ 19.8	3.2 $\pm$ 0.34
Myometrium	1117.6 $\pm$ 20.9	7.7 $\pm$ 0.25

### 3.5 RECEPTOR AND HORMONAL PROFILE OF PATIENTS IN THE FOLLICULAR AND LUTEAL PHASES OF THE MENSTRUAL CYCLE

#### Receptors profile (follicular phase n=3 & luteal phase n=2):

The mean  $\pm$ SEM value for ER in the myometria was  $20.0 \pm 0.6$  and  $33.6 \pm 2.5$  fmol/mg of protein in the fibroid; whereas the PR mean  $\pm$ SEM value were  $13.2 \pm 3.4$  and  $17.8 \pm 1.3$  fmol/mg of protein respectively, for the patient in the follicular phase. For patient in the luteal phase the mean  $\pm$ SEM value ER in myometria was  $17.4 \pm 0.5$  and  $22.7 \pm 4.3$  fmol/mg of protein in the fibroids. PR values were  $13.8 \pm 5.2$  and  $13.9 \pm 1.3$  fmol/mg of protein for myometria and fibroids respectively.

#### Hormonal profile (follicular phase n=3 & luteal phase n=2):

The mean  $\pm$ SEM value for  $E_2$  in the myometria was  $1169.0 \pm 19.0$  pmol/g tissue and  $650.0 \pm 30.5$  pmol/g tissue in the fibroids whereas  $P_4$  mean  $\pm$ SEM values were  $6.8 \pm 0.2$  nmol/g tissue and  $1.9 \pm 0.1$  nmol/g tissue respectively for patients in the follicular phase. For patients in the luteal phase  $E_2$  mean  $\pm$ SEM was  $1024.2 \pm 31.0$  pmol/g tissue and  $525.8 \pm 27.9$  pmol/g tissue in the fibroids. The  $P_4$  values were  $8.6 \pm 0.2$  nmol/g tissue and  $4.6 \pm 0.1$  nmol/g tissue in the myometria and fibroids respectively.

The receptor and hormone results in the fibroid and myometria for patients investigated during the follicular and luteal phases are compared in Table 4 (see results in Appendix VIII).

Table 4: ER, PR, E<sub>2</sub> and P<sub>4</sub> in the follicular and luteal phases of the menstrual cycle. (prot = protein and tis = tissue)

	Mean $\pm$ SEM fm/mg prot	Mean $\pm$ SEM fm/mg prot	Mean $\pm$ SEM pm/g tiss.	Mean $\pm$ SEM nm/g tis
<b>Follicular phase (n=3)</b>	<b>ER</b>	<b>PR</b>	<b>E<sub>2</sub></b>	<b>P<sub>4</sub></b>
Fibroid	33.6 $\pm$ 2.5	17.8 $\pm$ 1.3	650.0 $\pm$ 30.5	1.7 $\pm$ 0.1
Myometrium	20.0 $\pm$ 0.6	13.3 $\pm$ 3.4	1169.0 $\pm$ 19.0	6.8 $\pm$ 0.2
-	-	-	-	-
<b>Luteal phase (n=2)</b>	<b>ER</b>	<b>PR</b>	<b>E<sub>2</sub></b>	<b>P<sub>4</sub></b>
Fibroid	22.7 $\pm$ 4.3	13.9 $\pm$ 1.3	525.2 $\pm$ 27.9	4.6 $\pm$ 0.1
Myometrium	17.4 $\pm$ 0.5	13.8 $\pm$ 5.2	1024.2 $\pm$ 31.0	8.6 $\pm$ 0.2
-	-	-	-	-

From the results obtained for patients investigated in the follicular and luteal phases of the menstrual cycle the ER and PR were as usual higher in the fibroids compared to the normal myometria. Also the E<sub>2</sub> and P<sub>4</sub> were lower in the fibroid compared to the myometria. However, the total ER and PR in the uterine tissue (fibroid and myometrium) were higher in the follicular phase compared to the luteal. The total E<sub>2</sub> was also higher in the follicular phase. On the contrary, the total P<sub>4</sub> was lower (Table 4).

### 3.6 RESULTS OF HISTOLOGY OF MYOMETRIAL AND FIBROID TISSUES.

The histology of the uterine tissues (myometrium and fibroid) was done in order to exclude patients with fibroids co-existing with cancers and degenerative changes. The myometrial specimens of the 20 patients showed bundles of smooth muscle in normal arrangement. Likewise, the fibroid specimens showed whorling bands of spindle shaped smooth muscle cells in normal fashion. The histology of these specimens are shown in Plates I (normal myometrium), II (circumscribed fibroid nodole), III (whorling bands of spindle shaped smooth muscle cells in fibroid), IV (fascicles of smooth muscle cells in fibroids).



PLATE I: Normal myometrium showing bundles of smooth muscle in normal arrangement.

PLATE II: A well circumscribed nodule of closely packed bundles of smooth muscle in a fibroid. Notice how the normal myometrium has been pushed to the side (lower left).

PLATE I. x40

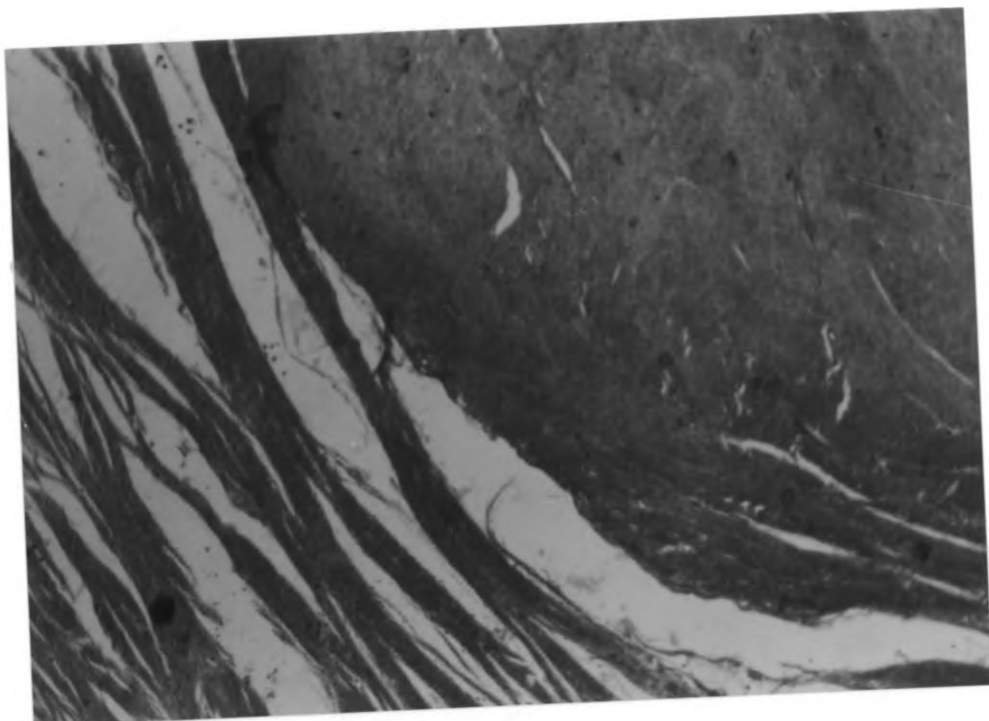


PLATE II. x40

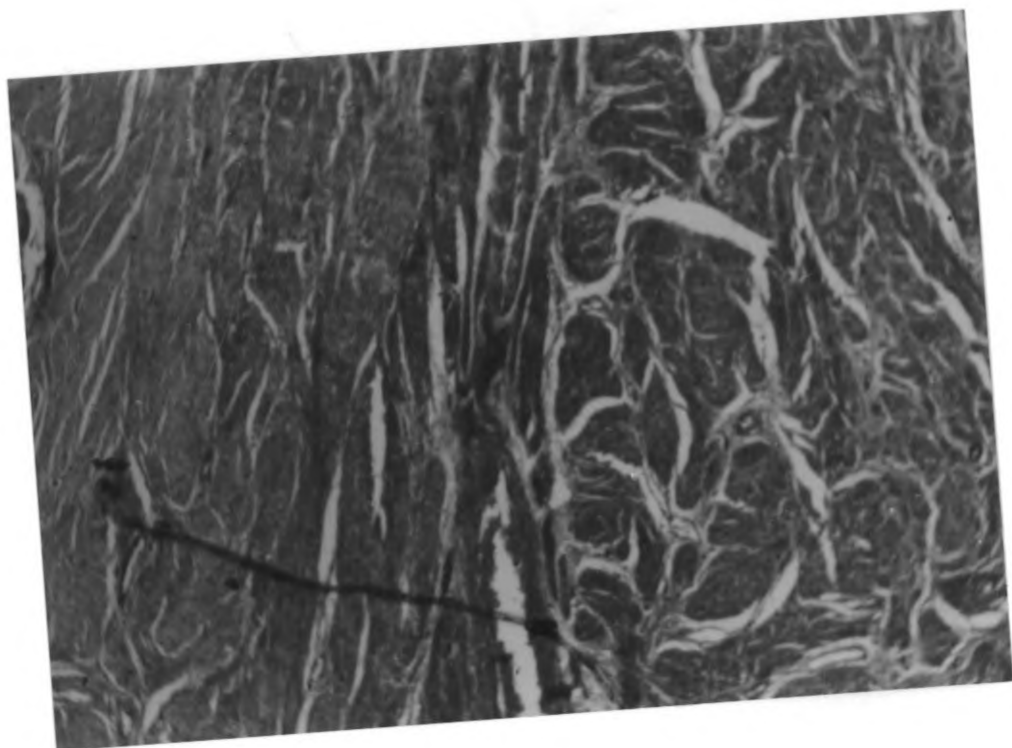
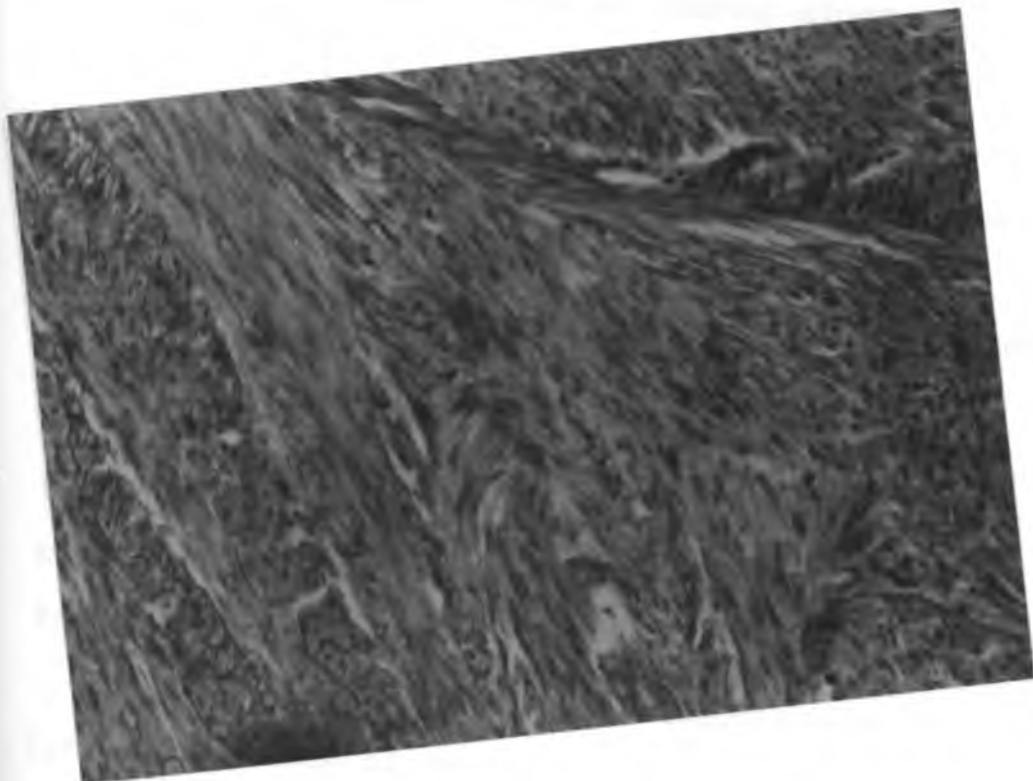


PLATE III: Whorling bands of spindle shaped smooth muscle cells  
leiomyoma.

PLATE III. x 100



## CHAPTER FOUR

### DISCUSSION

#### 4.1 ESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATIONS IN THE LEIOMYOMATA AND NORMAL MYOMETRIA.

In the quantitative estimation of receptor concentrations in steroid dependent tissues, physiological and analytical factors have been found to affect such measurements. Such factors include the race (Sadan et al. 1988), age (Roth, 1979) and menstrual status (Soules and McCarty, 1982) of the patient as well as exposure to hormonal agents. Hence in this study the receptor concentrations in the uterine leiomyomata were described in comparison to corresponding paired concentrations in the non-myomatous myometria from the same patient.

However, a careful patient selection was still done to ensure better estimates of the receptor levels. The patients used were pre-menopausal black females and age group 31-42 years. Difficulties were experienced in obtaining large sample sizes for patients in the proliferative and secretory phases of the menstrual cycles because most of the patients booked for hysterectomy had menstrual irregularity. Of the 20 patients studied 75 percent had menstrual irregularity, 15 percent were in the proliferative phase

while 10 percent in the secretory phase. All the patients had not been exposed to hormonal agents prior to surgery.

From the results described in Chapter Three, the uterine leiomyomata contained significantly higher levels of ER (147.6% :  $P < 0.001$ ) compared to the adjacent normal myometria. The PR levels in the uterine leiomyomata were also significantly higher (178.7% :  $P < 0.001$ ) than corresponding levels in the myometria. Hence the general observation drawn from these concentrations is that the adjacent normal myometria contain less receptors (ER & PR) than the leiomyomata. These findings were in agreement with previous study by Wilson et al. (1980) and also in agreement with recent work done by Potgieter et al. (1995) and Fernandez-Montoli et al. (1995).

Findings in this study have been described in comparison with such recent findings. Potgieter et al. (1995) working in South Africa reported significantly higher levels of ER and PR in the myomatous uteri compared to the normal myometria of control groups. In their work, Potgieter et al. (1995), observed that the ER range in the normal myometria of the control group was 54 - 92 fmol/mg cytosolic protein and mean  $\pm$ SEM was  $72.6 \pm 13.4$  while the range in the myomatous uteri in the experimental group was 94 - 293 fmol/mg and mean  $\pm$ SD of  $184.1 \pm 56.9$ . The PR range in the normal myometria was 490 - 966 fmol/mg with a mean  $\pm$ SD of  $79.3 \pm 223.0$  whereas the myomatous uteri had a range of 538 - 1927 fmol/mg and mean  $\pm$ SD was  $1050.2 \pm 370.5$ . They concluded that the leiomyomata appeared to contain significantly higher levels of ER (153.6% :  $P < 0.0001$ ) and PR (32.7% :  $P < 0.05$ ) than normal myometria.

Fernandez-Montoli et al. (1995) in a study in Spain showed a mean  $\pm$ SD value 7.25  $\pm$ 5.18 fmol/mg protein of ER in the myometria as compared to 14.66  $\pm$ 7.79 fmol/mg protein in the leiomyomata. The mean  $\pm$ SD value of PR was 68.38  $\pm$ 62.55 fmol/mg protein in the myometria and 216.90  $\pm$  206.67 fmol/mg protein in the leiomyomata. They also showed that ER and PR levels were higher in the myomatous uteri compared to the normal myometrium. Hence the general and popular opinion is that ER and PR levels are higher in the leiomyomata compared to normal myometrium.

In the present study though the ER and PR concentrations (compared within the same patients) were higher in the leiomyomata than in the myometrium, the ER levels in the myometrium and leiomyomata were higher than corresponding levels of PR in the same tissues. But the PR levels in the leiomyomata were raised to the same extent as the corresponding ER levels. This was contrary to results obtained by Potgieter et al. (1995), who reported higher levels of PR in the myometria and leiomyomata than corresponding ER in similar tissues and their PR levels in the leiomyomata were not raised to the same extent as corresponding ER levels. Also the results from Fernandez-Montoli et al. (1995) showed that though PR levels were higher than ER levels in corresponding myometria and leiomyomata, some individuals had much lower PRs than ERs. However, the differences in the levels of ER and PR in these three studies could arise from the difference in sample size, methodology (fibroid and myometrium from different or same patients), and from the fact that the phase of menstrual cycles were not controlled.

The higher levels of ER and PR in the fibroids could explain the occurrence of these tumours during the childbearing life and their increase in size during pregnancy, since these are physiological states in which circulating levels of sex steroid hormones are relatively high. This also explains the decrease in size of these tumours with GnRH-a therapy, whereby circulatory levels of estradiol are reduced to postmenopausal levels.

The high levels of receptors in the fibroids determine the capacity of the tumours to retain the hormones and could indicate a degree to which the same i.e fibroids are sensitive to the hormone action, and hence can synthesize specific proteins with enzyme activity in the target cells. This is in agreement with the speculation made by Potgiarter et al. (1995) that the regulation of the physiological action of both estrogen and progesterone is dependent on the relative concentrations of both ER and PR receptors.

#### **4.2 ESTRADIOL AND PROGESTERONE LEVELS IN THE LEIOMYOMATA AND NORMAL MYOMETRIA**

The results described in Chapter Three show that the adjacent normal myometria contained significantly higher levels of  $E_2$  (18.1% :  $P < 0.001$ ) compared to the leiomyomata. The  $P_4$  levels were also significantly higher (240.6% :  $P < 0.001$ ) in the adjacent normal myometria compared to the leiomyomata. Figures 9, 10 and 11 show



the comparative levels and means  $\pm$ SEM of  $E_2$  and  $P_4$  in the myometrium and leiomyomata. The observations drawn from these levels is that the adjacent normal myometria contain more of the free hormones ( $E_2$  &  $P_4$ ) than the leiomyomata. This is in agreement with findings of Farber, (1972) who had found out that the specific high affinity steroid hormone in a tissue determines its capacity to retain the hormone and indicate a degree to which the target tissue is sensitive to the hormone action. Hence the fibroid tissues bind more of the steroid hormone than does the adjacent normal myometrium. Jorge et al. (1990) also reached a similar conclusion when they showed that estrogen concentrations were higher in the leiomyomata than the myometrium.

Observations have shown that although the levels of estrogen and progesterone are different in the leiomyomata and myometrium, the circulating levels of these hormones are normal in patients with leiomyomata compared to control groups (Potgieter et al. 1990). Most scientific reports have tended to support the involvement of steroid hormones in the uterine fibroids, but there is little scientific evidence to the fact that these tumours are steroid hormone dependent. Work done to demonstrate this dependency has been criticized for reasons that fibroids in the human were found to be different from those in other animals, like the pig and the cow (Vollenhoven, 1990). With the lack of animal models, treatment modalities are being used to demonstrate the involvement of steroid hormones in fibroids. Treatment of leiomyomata with GnRH-agonists e.g. leuprolide acetate reduces serum  $E_2$  concentrations to post-

menopausal levels, and results in the shrinkage of the leiomyomata. Graigner et al. (1993) had concluded that the suppression of leiomyomata growth by GnRH-agonist was mediated by a decrease in estrogen receptors. Treatment with anti-progesterone RU486 (Murphy et al. 1993) had shown a decrease in fibroid size and the tissue examined after hysterectomy showed a significantly decreased PR content.

The above workers i.e Graigner et al. 1993 and Murphy et al. 1993, are in agreement with similar conclusions reached by Friedman et al. (1989) when they showed that a decrease in uterine size is dependent on the hypoestrogenic state which follow down regulation of the pituitary GnRH receptors and desensitization of gonatropes. Therefore the involvement of steroid hormones ( $E_2$  &  $P_4$ ) and steroid hormone receptors (ER & PR) in the etiopathogenesis has been scientifically established.

#### **4.3 RECEPTOR AND HORMONAL PROFILE OF PATIENTS INVESTIGATED DURING THE FOLLICULAR AND LUTEAL PHASES OF THE MENSTRUAL CYCLE.**

From the results represented in Table 4 in Chapter Three, higher levels of ER and PR were observed in the fibroids compared to the normal myometria in patients investigated during the follicular and luteal phases of the menstrual cycle. However, the total ER and total PR (i.e in the fibroid and myometrium) were higher in the follicular phase compared to the luteal. The total  $E_2$  in the follicular phase was higher than the same in the luteal

phase. On the contrary the total P<sub>4</sub> was higher in the luteal compared to the follicular phase.

It has been stated that a definite biochemical and physiological link exists between ER and PR; whereby PR levels appear to be stimulated by estrogens, while the ER and PR levels are down regulated by progesterone (Potgiarter et al. 1995). This could explain the low levels of total ER and total PR during the luteal phase wherein the P<sub>4</sub> levels were high, but fails to explain the high levels of both ER and PR during the follicular phase wherein the E<sup>2</sup> levels were high. A probable postulate that can be made from the observation of hormone and receptor concentrations among patients investigated in the two phases of the menstrual cycle is that the regulation of the physiological action of both estrogen and progesterone is dependent on the relative concentrations of both ER and PR receptors. Such a postulation would be limited by the fact that the sample sizes were small and the menstrual status of these patients were determined only by recording their menstrual histories.

## 5. CONCLUSIONS

Findings in this study show that the concentration of the sex steroid receptors (ER and PR) were significantly higher in the leiomyomata compared to the adjacent normal myometria of black Kenyan women. On the contrary the levels of the sex steroid

hormones ( $P_4$  and  $E_2$ ) were lower in the leiomyomata compared to the myometria in the same population. The total ER in the uterine tissues (fibroid and myometria) were higher than the total PR in same tissues, and the total  $P_4$  levels were higher than total  $E_2$ . For patients investigated during the different phases of the menstrual cycle the total ER and total PR were higher during the follicular phase compared to the luteal phase. The total  $E_2$  was also higher during the follicular phase as opposed to the total  $P_4$  which was lower in the same but higher in the luteal. However, observations during the different phases of the menstrual cycle were limited by the sample size.

From the above conclusions it is postulated that the relative proportions of  $E_2$ ,  $P_4$ , ER and PR in the individual patients uterine tissue may be important in the pathogenesis of fibroids in the black population in Kenya. It is further suggested that the treatment and management of the problem should involve manipulations of sex steroids and their receptors. Also research should continue in order to identify a nonsurgical means of treating this disease. This would be an important public health initiative especially in the black (negroid) population where fibroids occur at a higher incidence, surgical complications commoner, and childbearing the center of matrimonial harmony. In the clinical management of this disease the importance of early diagnosis and detection of high risk groups should be emphasized since current treatment modality i.e chemotherapy only reduces uterine size.

It is hoped that, besides the above recommendations, this study would help in resolving some of the controversies surrounding the levels of sex steroid hormones and their respective receptors in the leiomyomata and the normal myometria and provide information regarding this disease in the black population. This should also enable clinicians working in this population to appreciate more the use of chemotherapy in the management of uterine fibroids.

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APPENDIX I

Proforma form, for socio-demographic and reproductive characteristics of patients investigated

1. Name or in-patient number .....
2. Age .....
3. Date and time of operation .....
4. LMP .....
5. Menstrual patterns in the last 3 months .....
6. Parity .....
7. Drug treatment .....
8. Size of uterus .....
9. Size of fibroid .....
10. Co-existing operative abnormality .....
11. Histological aspects of the tissues .....

## APPENDIX II

Sample size determination

Determination of sample size for comparison of biological means:

$$N = \frac{(U + V)^2 (S_1^2 + S_2^2)}{(M_1 - M_2)^2}$$

(Handouts from: Workshop on Randomized Clinical Trials; Design Methodology and Analysis Family Health International, 1991).

Where N = Sample size

U = 1 sided deviate

V = 2 sided deviate

S<sub>1</sub> + S<sub>2</sub> = standard deviations

M<sub>1</sub> + M<sub>2</sub> = difference in means

A previous study has shown:

mean of ER = 7.25 SD = 5.18 for myometrium

mean of PR = 14.66 SD = 7.79 for fibroid

where U = 1 sided deviate and power 90%

$$= 1.28$$

where V = 2 sided deviate 5% significance

$$= 1.96$$

therefore substituting in the formula above:

$$N = \frac{(1.96 + 1.28)^2 (5.18^2 + 7.79^2)}{(14.66 - 7.25)^2} = 16.7$$

Sample size = 17

APPENDIX III.

Levels of statistical significance of results of ER and PR in the fibroids compared to the myometria

This was done using the student-t-test formula described in statistical procedures in Chapter 2:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s_1^2 + s_2^2}}$$

Applying the above formula for ER in myometrium and fibroid as in table 5 where:-

$$\bar{x}_1 = 28.2$$

$$\bar{x}_2 = 19.1$$

$$n = 20 \quad (n_1 = n_2)$$

$$S.E.M_1 = 1.6$$

$$S.E.M_2 = 0.4$$

$$\text{But } SD = S.E.M \times \sqrt{n}$$

$$\Rightarrow SD^2 = (S.E.M \times \sqrt{n})^2$$

$$\Rightarrow SD^2 = S.E.M^2 \times n$$

Where SD = standard deviation

n = Sample size

$$\Rightarrow S_1^2 = 51.2$$

$$\& s_2^2 = 3.2$$

Substituting these values in the formula above  $t = 5.515$ .

From the tables of "Distribution of t Probability" abridged from Table III of Fisher and Yates by biological science curriculum study (Norman, Schrot, Balch et al. 1970), a P-value of 0.05 or less is taken as showing significant difference. Hence for the t of 5.515 calculated above the P-value is less than 0.001, expressed mathematically as  $P < 0.001$ . Therefore, a significant difference exist between the ER levels in the myometrium and fibroid.

Applying the same formula for PR as per table 5 :

$$\Rightarrow t = 10.137$$

From the table of "Distribution of t Probability the P-value is

less than 1.001 i.e.  $P < 0.001$ . Hence there is a significant difference between the two PR levels.

#### APPENDIX IV.

Level of statistical significance of values of  $E_2$  and  $P_4$  in the fibroids compared to the myometria.

The statistical analysis for hormonal parameters were performed using the "Student-t-test formula.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2 + s_2^2}{n}}}$$

Applying the above formula for  $E_2$  levels in fibroid and myometrium as per Table 3.7 where:-

$$\bar{x}_1 = 1117.6$$

$$\bar{x}_2 = 616.9$$

$$n = 20 (n_1 = n_2)$$

$$s_1^2 = 8736.2$$

$$s_2^2 = 7840.8$$

Substituting value in the equation above:

$$t = 17.391$$

From the tables of "Distribution of t Probability" the P-value is less than 0.001 i.e.  $P < 0.0001$ .

The same formula when applied for  $P_4$  levels in fibroid and Myometrium as per Table 3.7;

$$\Rightarrow t = 1.714$$

And from the tables of "Distribution of t Probability" the P-value is less than 0.001 i.e  $P < 0.001$ .

Hence there is a significant different between the levels of  $P_4$  in the fibroid and myometrium.

APPENDIX V:

Cytosol dilutions (with TEDG buffer) versus percentage of tracer bound to receptors in the myometria and fibroids.

Dilutions of cytosol (V/V)	Myometrium (% tracer bound)	Fibroid (%tracer bound)
1:0	0.9	7.5
1:1	0.7	4.8
1:2	0.4	2.4
1:4	0.2	1.0
1:8	0.2	0.7
1:16	0.1	0.7

Cytosol dilutions versus percentage tracer bound to fibroid receptors at different tracer concentrations.

Cytosol dilutions	Myometrium 3,500cpm (3.5nCi)	Fibroid 15,000cpm (15nCi)
1:0	3.5 %	7.5 %
1:1	2.9 %	4.8 %
1:2	1.5 %	2.4 %
1:4	0.4 %	1.0 %
1:8	0.2 %	0.7 %
1:16	0.08 %	0.7 %

APPENDIX VI. ER and PR levels (fibroids & Myometria) n=20

Patient ID No.	ER in Myometrium (f moles/mg protein)	ER in fibroid (f moles/mg protein)	PR in Myometrium (f moles/mg protein)	PR in fibroid (f moles/mg protein)
1	19.3	28.3	9.8	15.9
2	16.3	18.9	7.5	13.7
3	21.1	37.3	10.4	18.9
4	18.1	27.3	8.9	14.8
5	17.9	18.4	8.6	12.6
6	18.4	26.5	9.7	16.8
7	17.5	20.8	8.5	16.9
8	19.2	33.5	9.2	19.3
9	16.6	22.6	8.1	13.3
10	20.2	35.5	10.1	20.3
11	17.4	19.8	8.3	11.9
12	22.0	36.9	10.6	18.9
13	17.6	20.4	9.1	19.3
14	19.3	28.2	9.6	17.5
15	21.9	34.3	10.9	17.2
16	22.5	38.2	11.2	21.0
17	19.6	29.3	9.9	15.1
18	16.9	26.9	8.2	13.3
19	17.7	23.7	8.7	16.8
20	22.3	39.3	11.3	21.9



APPENDIX VII. E<sub>2</sub> and P<sub>4</sub> levels (fibroids and myometria) n=20

Patient ID No.	E2 in Myometrium (pico moles/gram tissue)	E2 in Fibroid pmol/g tissue	P4 in Myometrium (nano moles/gram tissue)	P4 in Fibroid nmol/g tissue
1	1139.3	611.2	8.2	4.5
2	972.5	469.5	5.9	1.2
3	1202.8	710.3	6.9	1.9
4	1023.6	601.9	6.8	1.7
5	1055.1	553.6	8.8	4.6
6	1103.2	642.0	7.4	2.3
7	1096.9	544.1	6.7	1.6
8	1137.0	627.4	7.1	2.2
9	987.7	479.3	6.1	1.5
10	1196.5	687.5	8.6	4.7
11	1067.1	599.7	8.8	4.8
12	1211.2	703.5	9.1	4.9
13	1012.4	526.0	7.0	2.1
14	1144.0	623.1	7.7	2.4
15	1206.9	714.8	8.9	4.8
16	1307.8	789.3	9.3	5.1
17	1167.1	612.6	6.3	1.7
18	993.2	497.9	8.4	4.5
19	1089.4	607.1	6.7	1.7
20	1237.3	736.5	9.1	5.0

APPENDIX VIII.

(a) ER and PR (fm/mg protein) in the fibroids and myometria of patients in the follicular and luteal phases of the menstrual cycle.

Follicular Phase n = 3				
Patients ID NO	ER in Myometrium fm/mg protein	ER in Fibroid fm/mg protein	PR in Myometrium fm/mg protein	PR in Fibroid fm/mg protei
3	21.1	37.9	10.4	18.9
8	19.2	33.5	9.2	19.3
17	19.6	29.3	19.9	15.1

Luteal Phase n = 2				
5	17.9	18.4	8.6	12.6
18	16.9	26.9	18.9	15.1

(b)  $E_2$  and  $P_4$  in the fibroids and myometria of patients in the follicular and luteal phases. ( $E_2$  in nm/g tissue and  $P_4$  in pm/g tissue).

Follicular phase n = 3				
Patient ID No.	$E_2$ in Myometrium	$E_2$ in Fibroid	$P_4$ in Myometrium	$P_4$ in Fibroid
3	1202.8	710.3	6.9	1.9
8	1137.0	627.4	7.1	2.2
17	1167.1	612.6	6.3	1.7

Luteal phase n = 2				
Patient ID No.	$E_2$ in Myometrium	$E_2$ in Fibroid	$P_4$ Myometrium	$P_4$ in Fibroid
5	1055.1	553.6	8.8	4.6
18	993.2	497.9	8.4	4.5