ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

SERUM AND TISSUE PROLACTIN AND ITS RECEPTORS IN PATIENTS UNDERGOING MYOMECTOMY AND HYSTERECTOMY

PhD. Phil. Dissertation submitted to

Al-NAHRAIN UNIVERSITY

In partial fulfillment of the requirements
For the award of the degree of

DOCTORATE OF PHILOSOPHY IN CLINICAL BIOCHEMISTRY
BY

Rayah Sulaiman Salah Al-Deen Baban

Under The Guidance of
Prof. Dr. YAHYA Y. Zeki
&
Lecturer Dr. Shatha Al-Zuheiri

College Of Medicine
Al-Nahrain University
1987

DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY
COLLEGE OF MEDICINE
Year of Award: 2006

Abstract:

Background: Uterine leiomyomas, commonly referred to, as "fibroids" are benign tumors arising from the myometrial compartment of the uterus. They are typically well differentiated, have a relatively low mitotic index, and retain their smooth muscle phenotype. Uterine leiomyomas are the most common gynecologic neoplasm, occurring with a remarkable frequency in more than 70% of women at their reproductive age.

Several endogenous or environmental factors that modulate the risk for developing uterine leiomyoma affirm the hormone responsive nature of this disease. Obesity and age at menarche have been linked to an increased risk of uterine leiomyoma, while cigarette smoking, use of oral contraceptives containing progesterone, and parity have been identified as protective factors.

The female reproductive tract is known to be an extrapituitary source of the hormone prolactin. The endometrium, myometrium, and uterine leiomyomas (fibroids) all have been shown to secrete prolactin when cultured in vitro.

Prolactin (PRL) is a polypeptide (23 kDa) hormone of growth hormone/cytokine family. It is secreted mainly by the lactotrophs in the anterior pituitary gland and by several other tissues also at low concentrations. In the serum, PRL occurs in various molecular forms, including the physiologically active monomeric form, also called the little PRL (L-PRL; molecular weight, 23 kDa), the big PRL (B-PRL; molecular weight, 50-60 kDa), and the big big PRL (BB-PRL; molecular weight, 150-170 kDa), that is also called macroprolactin. L-PRL is usually the dominant form in the serum of healthy and hyperprolactinemic individuals. The exact structures of B-PRL and BB-PRL are not fully understood: B-PRL is thought to consist of oligomers of PRL, whereas BB-PRL has been suggested to consist of an immunoglobulin G (IgG) complex with one or two PRL molecules Hyperprolactinemia is mostly caused by a pituitary adenoma, but it may be also caused by macroprolactin. Because the big forms of PRL have a decreased bioactivity, they do not cause clinical symptoms of hyperprolactinemia.

It is known that the classical effects of PRL are on the development of the mammary gland and lactation. In addition to that, reproduction as well as other effects of PRL is initiated by the interaction of the hormone with specific, high-affinity receptors located on the plasma membrane and widely distributed in a number of tissues. Different biochemical approaches (cross-linking, immunoprecipitation, or immunoblot analysis with monoclonal antibodies) have shown that the prolactin receptor has a relative molecular

weight of ~40,000 and is apparently not linked by disulfide bonds to itself or to other subunits. Prolactin receptor (PRLR) level is differentially regulated in different tissues. It increases during pregnancy, and is markedly stimulated by estrogens. Prolactin plays a major role in the regulation of its own receptor, including both up- and down regulation, depending on the concentration and duration of exposure to PRL.

Objectives: To study the PRL profile with its molecular weights in serum, leiomyomas, and patients myometrium compared with the PRL profile of a normal myometrium. Also to evaluate the relation between the leiomyomas PRL and their sizes in addition to their correlation with PRL in sera.

Subjects and methods: The circulating prolactin of the patient group (n=53) as well as their tissues prolactin [(leiomyomas and myometrium)] and [normal myometrium] of the control group (n=40) was assayed using the Prolactin Kit (Biomérieux). Prolactin profile was detected using the polyethylene glycol 8000 precipitating method to separate the big big prolactin from the monomeric and the big prolactin isoforms. Disk gel electrophoresis technique was used to confirm the prolactin isoforms and to calculate their molecular weight. While the prolactin receptor was detected in tissue sections by using an immunohistochemical staining method with monoclonal antibody [BIOCARE detection kit].

Results: Mean age of the patient group was 39.9±5.5 years while that of the normal control group was 32 years. All leiomyomas patients were obese with body mass index (BMI) mean 28.4±4.7 kg/m2. Their mean age of menarche was 13.1±0.6 years. They weren't smokers neither oral contraceptive users. Eighty-three percent of patients were married and the rest (16.98%) were single. Parity for the married patients was ranging from nulliparous to 5 and the highest percentage was among those with 1-4 parity (70.5%).

The prolactin mean for patient's serum was 143.1±106.9 ng/ml, leiomyoma was 18.2±14.0 ng/ml and for patients myometrium was 8.0±3.3 ng/ml respectively, while the mean for the normal control myometrium was 6.7±2.6 ng/ml.

A highly significant difference was found between the leiomyoma prolactin and patient's myometrium prolactin as well as between the leiomyoma prolactin and the normal myometrium prolactin ($P<0.0001$), while no significance was found between patient's myometrium and the normal myometrium prolactin.

Also a high significance ($P<0.0001$) was found between the patients serum prolactin and their leiomyoma prolactin, leiomyoma size, and between the serum prolactin/leiomyoma prolactin ratio with the leiomyoma size, while a significant value ($P<0.05$) was found between leiomyoma prolactin and their sizes.

In this study the predominant prolactin isoform found was the monomeric prolactin. In patient's serum, the monomeric prolactin was found with two molecular weights, 24.9±9.7 and 16.5±4.5 kDa, while in their tissues (leiomyoma and myometrium) they were 16.5±5.0 and 16.7±6.9 kDa. The mean molecular weight for monomeric prolactin isoform in normal myometrium of control group was 16.7±5.7 kDa.

The second predominant prolactin isoform was the big prolactin. It was found with 71.6±15.7 kDa in patient's serum while it was 70.5±11.2 and 78.5±14.9 kDa in their leiomyoma and myometrium respectively. The normal control myometrium had big prolactin molecular weight of 74.4±13.2 kDa. Only one sample of patient's serum had big big prolactin isoform with molecular weight 201.5 kDa.

The immunohistochemical staining of leiomyoma sections revealed brown spots, which indicate the presence of prolactin receptor on the membrane of leiomyoma.

Conclusion: Age, obesity, and age at menarche were the risk factors associated with developing leiomyoma, while increasing parity did not decrease this risk as was known.

Serum prolactin level was increased in patient with uterine leiomyoma with a significant positive correlation with the leiomyoma size and its prolactin produced from it

Approved by
College of Medicine / Al-Nahrain University
as a thesis for the degree of

Doctor of Philosophy

In

Clinical Biochemistry

Professor
Dr. Hikmat A. Hatam

Dean
College of Medicine / Al-Nahrain University

Seal
Higher Studies Registration
College of Medicine / Al-Nahrain University

Thesis Chapters: (List of headings used in the thesis, like Introduction, Background, Material and Methods, Observations,)

Dedication

To the spirit of my *father*
To my dearest *mother*
To my only *brother*

We certify that this thesis was prepared under our supervision at College of Medicine / Al-Nahrain University, as a partial fulfillment of the requirements for the Degree of Doctor Philosophy in Clinical Biochemistry.

Professor
Dr. Yahya Y. Zeki
Supervisor

Senior Lecturer
Dr. Shatha Al-Zuheiri
Consultant

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Professor
Dr. Ghassan A. Al-Shammaa
Chairman
Department of Physiological Chemistry

We, the Examining Committee, certify that we have read this thesis and have examined the student ***Rayah S. Baban*** in its contents and, at our opinion, it is adequate with standing: Excellent as a thesis for the degree of Doctor of Philosophy in Clinical Biochemistry.

Professor
Dr. Ghassan Al-Shammaa
Chairman

Professor
Dr. Sajedah A.H. Khalil
Member

Professor
Dr. Abdul-Wahab R. Hamad
Member

Assistant Professor
Dr. Maha Al-Bayati
Member

Assistant Professor
Dr. Ali M. Al-Gharawi
Member

Acknowledgements

Praise to ***God***, the lord of all that be, the Merciful.

I would like to express my sincere thanks and my appreciation to my supervisors, ***Professor Dr. Yahya Y.Z. Farid,*** for his supervision of my study and for his cooperation during the time of writing. I am greatly indebted to him for his suggestions and valuable remarks and his assistance.

I wish to express my deepest thanks to my second supervisor ***Dr. Shatha Al-Zuhairy*** for her kind opinions, support and introducing facilities throughout this work.

I am boundlessly grateful to ***Professor Dr. Ghasssan Al-Shammaa*** Chairman of Department of Physiological Chemistry, College of Medicine, Al-Nahrain University for his generous help and his support during the time of my project.

In surly I can not forget ***Dr. Sahar Halabia*** for given me the idea of this study and her help.

Thanks are due to ***assistant professor Dr. Alaa Ghani*** Chairman of Department of Pathology for his generous help during the time of my project.

Extreme thanks to the laboratory staff in the Al-Khadimiya Teaching Hospital for their gentle help.

Greeting and thanks to ***Dr. Maysaloun M. Al-Ani*** for assisting me in the statistical aspects of this project.

Thanks and appreciation for ***Dr. Huda J. Al-Azzawi*** for her support and help during my study.

A special thanks is extended to ***Dr. Rhana Y. Zaki*** for helping me in many things during my study.

Thanks for all members in the physiological Chemistry Department for their help.

I am boundlessly grateful to my mother for her patience and encouragement in order to finish my graduate studies.

Rayah

Serum and Tissue Prolactin and its Receptor in Patients undergoing Myomectomy or Hysterectomy

A Thesis Submitted to the
College of Medicine / Al-Nahrain University
in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy

In

Clinical Biochemistry

By
Rayah Sulaiman Salah Al-Deen Baban
B. Sc. in Chemistry (Baghdad University 1985)
M. Sc. in Biochemistry (Baghdad University 1992)

Professor
Dr. Yahya Y. Zeki
Supervisor

Senior Lecturer
Dr. Shatha Al-Zuheiri
Consultant

October 2006

Rhamadan 1427

List of Contents

List of Tables

List of Figures

List of Abbreviations

-/-	gene-deficient mice
B	big
BB	bigbig
BMI	body Mass Index
CIS	cytokine inducible SH2-domain-containing protein
ER	estrogen receptor
GH	growth hormone
GHR	growth hormone receptor
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
hPRL	human prolactin
IGF-I	Insulin-like growth factor I
JAK	janus kinase
Kb	kilo base pair(s)
LM	leiomyoma
mRNA	messenger ribonucleic acid
MWt	molecular weight
NMR	nucleic magnetic resonance
OC	oral contraceptive
P	probability
PEG	polyethylene glycol
PL	placental lectogen
PR	progesterone receptor
PRL	prolactin
PRLR	prolactin receptor
r	correlation coefficient
SDS	sodium dodecyl sulphate
SOCS	suppressors of cytokine signaling
Stat	signal transducer and activator of transcription
xg	relative centrifuge force

Chapter One

Introduction

Introduction and Literature Review:

1.1 Uterine leiomyomas:

Uterine fibroids are tumors made of connective tissue and smooth muscle. They grow slowly within the wall of the uterus or attach to the uterine wall. Most fibroids are non cancerous, although in some rare cases they may become cancerous. This occurs in less than 1% of fibroids (McWilliams 2005) .

These fibroids may be as small as a pea or as large as a grapefruit. As the fibroid grows, the uterus may become deformed or pushed aside. When the uterus is deformed or blocked by a growth, the resulting pressure may cause symptoms in the bladder or intestine, such as increased urination, constipation, or pain. In pregnant woman, fibroids can cause uterine pain and even increase the risk of miscarriage. Other terms used for a uterine fibroid are leiomyoma or myoma of the uterus.

Leiomyomas are classified by their location in the uterus. Subserosal leiomyomas are located just under the uterine serosa and may be pedunculated (attached to the corpus by a narrow stalk) or sessile (broad-based). Intramural leiomyomas are found predominantly within the thick myometrium but may distort the uterine cavity or cause an irregular external uterine contour. Submucous leiomyomas are located just under the uterine mucosa (endometrium) and, like subserosal leiomyomas, maybe either pedunculated or sessile. Tumors in subserosal and intramural

locations comprise the majority (95%) of all leiomyomas; submucous leiomyomas make up the remaining (5%) (Chamberlain 2000).

Uterine leiomyomas, or fibroids, are the most common tumors of women in the United States; probably occurring in the majority of women by the time they reach menopause and becoming clinically significant in about one-third of these women. Despite their prevalence, little attention has been directed toward the causation and pathogenesis of fibroids until recent years because of the rarity of their malignant transformation. Regardless of their generally benign neoplastic character, uterine fibroids are responsible for significant morbidity in a large segment of the female population. The clinical effects of these tumors are related to their local mass effect, resulting in pressure upon adjacent organs, excessive uterine bleeding, or problems related to pregnancy, including infertility and repetitive pregnancy loss (Haney 2000). As a consequence of these local pressure effects and bleeding, uterine fibroids rank as the major reason for hysterectomy in the United States, accounting for approximately one-third of all hysterectomies (Wilcox *et al.* 1994), or about 200,000 hysterectomies per year (Gambone *et al.*1990).

Fibroids are usually found during routine pelvic exams. If the patient has severe menstrual symptoms or other pelvic problems, an ultrasound scan maybe recommended determining the cause of the problems.

For fibroids that require treatment, gynecologist may suggest a myomectomy or hysterectomy. A myomectomy is a type of surgery used to remove the fibroids without having to remove the uterus. It is

usually done when the possibility of having children after the surgery is planned. The chance of fibroids recurrence is dependent on the number of fibroids that has developed in the first place. It appears that the more fibroids a woman's uterus develops, the more likely they are to recurrence after a myomectomy. In a hysterectomy, the surgeon removes the uterus. This making the possibility of fibroids recurrence zero. A myomectomy can be technically more difficult than a hysterectomy and can lead to greater blood loss (McWilliams 2005).

Considering the development of uterine leiomyomas, it is convenient to subdivide the factors that may be related to tumorigenesis into four categories: risk factors, initiators, promoters, and effectors.

1.1.1. Risk Factors Associated with Leiomyomas:

Risk factors are characteristics associated with a condition, generally identified by epidemiological studies. Knowledge of such predisposing factors may provide clues to the etiology of these tumors as well as to preventive measures. The occurrence of genetic aberrations in fibroid tumors is considered. Despite the abundance of cytogenetic investigations, uncertainty remains as to the primary or secondary nature of these genetic changes and their impact on the initiation and/or promotion of these tumors. The role of growth promoters of fibroids seems to belong in large part to the ovarian hormones estrogen and progesterone (Gordon *et al.* 2003).

Lauren *et al.* (2004) assessed the risk of uterine leiomyomata in relation to reproductive factors and hormonal contraception in a prospective cohort study of US Black women. After adjustment for age, body mass index, smoking, alcohol intake, and other reproductive covariates, the risk of ultrasound-or hysterectomy-confirmed uterine leiomyomata was inversely associated with age at menarche, parity, and age at first birth and positively associated with years since last birth. Overweight or obesity appeared to attenuate the inverse association between parity and leiomyomata. Current use of progestin-only indictable was inversely associated with risk. No consistent patterns were observed for other forms of hormonal contraception. Reproductive history is an important determinant of leiomyomata risk in pre-menopausal women.

1.1.1.1. Age:

An increase with age in the prevalence of fibroids during the reproductive years has been demonstrated by several epidemiologic studies (Wilcox *et al.* 1994; Marshall *et al.* 1997; Velebil *et al.* 1995). Ross *et al.* (1986) have shown a rapid increase in fibroid diagnoses among women in their forties. Whether the risk of new fibroids actually increases rapidly in women during their forties is not known. The observed increase could also result from increased growth of or increased symptomatology from, already existing fibroids, as well as from a greater willingness of women in the later reproductive years to have gynecologic surgery. If the likelihood of fibroid development and growth actually accelerates during the late reproductive years, hormonal factors associated with perimenopause may be important

modulators; alternatively, the apparent increase in the late reproductive years may simply represent the cumulative culmination of 20-30 years of stimulation by estrogen and progesterone.

1.1.1.2. Menarche:

There is a suggestion of slightly increased risk of fibroids associated with early menarche, although the risk has often not been statistically significant (Cramer *et al.*1995; Parazzini *et al.* 1988; Samadi *et al.* 1996). Marshall *et al.* (1998) have reported a significant inverse association between risk of fibroids and age at menarche; they said women who were 12 years of age at menarche and those who were < 10 years of age at menarche were at increased risk [relative risk (RR)_1.24], whereas women who were age ≥ 16 years at menarche were at lower risk (RR 0.68). The early onset of menstrual cycles may increase the number of cell divisions that the myometrium undergoes during the reproductive years, resulting in an increased chance of mutation in genes controlling myometrial proliferation. Sato *et al.* (2000) found that women with uterine leiomyomas more often exhibited an early normal menstrual cycle pattern, and concluded that early menstrual regularity may enhance leiomyoma growth in early reproductive life.

1.1.1.3. Parity:

Many factors that can modulate the risk of developing uterine leiomyoma have been identified, including parity. Epidemiological data on decreased risk of developing leiomyoma has been subjected to

different interpretations regarding whether pregnancy itself is protective or, as leiomyomas are a major cause of infertility, women that develop these tumors are less fertile and thus have lower pregnancy rates. Researchers have utilized an animal model genetically predisposed to uterine leiomyoma to investigate the potential protective effect of pregnancy on the risk of developing this disease. Female Eker rats that carry a mutation in the tuberous sclerosis 2 (Tsc-2) tumor suppressor gene develop uterine leiomyoma with a frequency of 65% when nulliparous. These animals were bred with intact or vasectomized males and tumor incidence determined after a single pregnancy (to confirm fertility) or multiple pregnancies over the lifetime of the animals. Females with multiple litters displayed a dramatic shift in tumor incidence and presentation. Tumor incidence decreased from 71% in single litter females to 10% in females that had multiple litters (average: five litters/animal) (Cheryl *et al.* 2001).

Studies that report changes in existing leiomyomas during pregnancy are not consistent. Some tumors grow, others shrink, but many show little change. (Ross *et al.* 1986; Lumbiganon *et al.* 1996). While epidemiological evidence indicates a protective effect of pregnancy for human leiomyoma, the mechanism(s) by which pregnancy exerts its protective effects is unclear. Pregnancy has been shown to modulate the incidence of other types of hormone-dependent tumors, although the mechanisms of this protective effect appear to operate at different levels in different cell types. In breast cancer, early pregnancy is the most protective, suggesting an effect on the

normal target cell population from which these tumors arise (Adami *et al.* 1998; Kelsey *and Bernstein* 1996; Colditz 1993). In contrast, in the endometrium, time of last pregnancy appears to be a more important determinant. In women with only a single pregnancy, the most protection is afforded to women having a late rather than early pregnancy. This suggests that the protective effect is acting against the nascent neoplastic/preneoplastic cell population (Parslov *et al.* 2000; Franchesschi 1998; Lambe *et al.* 1999). The fact that no protection against the development of uterine leiomyoma was afforded in a study with female Eker rats undergoing a single early pregnancy. Cheryl (2002) suggests that the protective mechanisms of pregnancy in the myometrium are more similar to the endometrium (*i.e.*, acting against nascent tumor cells) than the breast (protective differentiation).

Anderson *et al.* (1995) have suggested an interesting hypothesis in which leiomyoma cells were described as resembling a myometrial cell of pregnancy rather than a typical myometrial cell under the influence of the menstrual cycle. These similarities include increased levels of steroid hormone receptors, expression of insulin growth factor-I (IGF-I), and production of extracellular matrix proteins such as collagens type II and I. Furthermore, leiomyomas constitutively express high levels of connexin43 (contraction associated protein), the predominant connexin found in the myometrium during pregnancy (Anderson *et al.* 1993). Connexin 43 expression is negligible in myometrial cells during the normal menstrual cycle and early pregnancy; however, at term, connexin 43 becomes abundantly expressed under the influence of estrogen (Chow and Lye, 1994). This

confirms that leiomyomas not only share the characteristics of myometrial cells during pregnancy but also more specifically have the phenotype of these cells at parturition.

1.1.1.4. Obesity:

Several studies have found an association between obesity and an increased incidence of uterine leiomyomas. Risk of fibroids found to be increased approximately 21% for each 10-kg increase in body weight; similar results were obtained when the body mass index (BMI) was analyzed rather than weight (Ross *et al.* 1986). A significant increase occurs in the conversion of circulating adrenal androgens to estrone by excess adipose tissue. The hepatic production of sex hormone-binding globulin is decreased, resulting in more unbound physiologically active estrogen. Because almost all circulating estrogens postmenopausally are derived from metabolism of circulating androgens by peripheral tissues, including fat, these two mechanisms probably have more impact in postmenopausal than pre-menopausal women (Glass 1989).

In obese pre-menopausal women, decreased metabolism of estradiol by the 2-hydroxylation route will reduce the conversion of estradiol to inactive metabolites, which could result in a relatively hyperestrogenic state (Schneider *et al.* 1983).

1.1.1.5. Oral Contraceptives (OCs):

Oral contraceptives may play a role in the development or growth of leiomyomata. Some have found no association between the occurrence of fibroids and the use of OCs (Samadi *et al.* 1996), while

others have reported a reduction in risk of fibroids with OC use. They have observed in their study a consistent decrease in the risk of fibroids with increasing the duration of OC use (approximate 17% reduction in risk with each 5 years of use); this apparent protective effect was attributed to reduce exposure to unopposed estrogen due to the modifying effect of progestogens (Ross *et al.* 1986).

These conflicting findings with regard to the effect of OCs upon the growth of myomas may relate to the differing content of estrogen and the type of progestogen in each specific OC preparation (Cramer 1992). Group of researchers attempted to address this issue by analyzing the estrogen and progesterone content of each formulation. Although no conclusions could be drawn regarding the estrogens present, the authors found that the higher the dose of the progestogen norethisterone acetate, the lower the incidence of fibroids, in preparations containing the same quantity of the estrogen ethinylestradiol. In contrast, all preparations containing the progestogen ethynodiol diacetate were associated with an increased incidence of fibroids, regardless of the quantity present or the type or amount of the accompanying estrogen. The authors offered no explanation for the latter finding and stated that additional studies were needed for confirmation (Gordon *et al.* 2003).

1.1.1.6. Menopause:

A reduced risk of fibroids requiring surgery in postmenopausal patients could be due to tumor shrinkage in the absence of hormonal stimulus following the menopause (Samadi *et al.* 1996). A study have found a similar incidence of leiomyomas in both pre- and

postmenopausal patients (74 and 84%, respectively), although the postmenopausal leiomyomas were smaller and fewer (Cramer and Patel 1990). The estimated risk in postmenopausal patients could be reduced by selection bias because of a tendency toward a more conservative non-surgical, clinical approach in postmenopausal women (Parazzini *et al.* 1988).

1.1.1.7. Racial Differences:

Uterine fibroids are more prevalent in black women than white women. In a study of hysterectomy specimens, 89% of the black women and 59% of the white women had leiomyomas, which in black women were often larger, more numerous, and more symptomatic, and had developed at a younger age (Kjerulff *et al.* 1996). In another study, 95,061 pre-menopausal women with no history of uterine leiomyoma were followed prospectively and had an incidence rate of leiomyoma approximately 2-3 times greater among black women than among white women. Although there was a higher prevalence of risk factors, including a higher mean BMI, among black women in this study, these factors could not account for the excessive rate of uterine leiomyomata among pre-menopausal black women (Marshall *et al.* 1997).

1.1.1.8. Smoking:

Several studies have revealed a reduced risk of fibroids associated with current smoking, but not past smoking (Lumbiganon

et al. 1996; Samadi *et al.* 1996). Researches have reported in a study that the risk of uterine myomas requiring surgery reduced 50% in current smokers (Parazzini *et al.* 1996). The inverse correlation between smoking and fibroids has been commonly attributed to an anti-estrogenic effect of cigarette smoking, suggested by other epidemiologic associations of smoking, including a reduced risk of endometrial cancer, earlier natural menopause, and increased osteoporosis. Nicotine inhibition of aromatase reduces the conversion of androgens to estrone (Barbieri *et al.* 1986). Significantly higher serum levels of sex hormone-binding globulin have been found, resulting in less unbound physiologically active estrogen. These studies indicate that the hormonal metabolic effects of smoking are probably multifactoral (Daniel *et al.* 1992). In addition, smokers as a group consistently exhibit lower body weights than nonsmokers, possibly because of a lower efficiency of calorie storage and/or an increase in the metabolic rate (Wack and Rodin 1982). A lower body weight associated with a reduced risk of fibroids might be expected to be another indirect contributing mechanism through which smoking exerts an effect, but in another study, it was found that the effect of smoking was not changed by correction for BMI (Lumbiganon *et al.* 1996; Schwatz *et al.* 2000a).

1.1.1.9. Prolactin:

Although initially identified as a pituitary gland hormone, several studies have demonstrated that prolactin is also produced by uterine tissues, including the endometrium, myometrium, and uterine leiomyomas (Walters *et al.* 1983). The significance of prolactin production in leiomyomas is not yet well defined; however, interest in this hormone has been stimulated by the finding that prolactin acts as a mitogen for vascular smooth muscle (Sauro and Zorn 1991). In addition, in one study of myometrial and leiomyoma explants cultures; fibroid prolactin secretion was substantially greater than myometrial prolactin secretion (Rein *et al.* 1990). In another study researchers had found that estrogen enhanced the secretion of prolactin in fibroid tissue cultures, whereas progesterone exhibited a suppressive effect (Daly *et al.* 1984). Because leiomyomas are mitotically active during the luteal phase, the inhibition of leiomyoma prolactin production by progesterone tends to cast some doubt upon the role of this hormone in fibroid growth. However, in a recent study, treatment of leiomyoma and myometrial cell cultures with a prolactin-neutralizing antibody inhibited cell proliferation, leading the authors to conclude that prolactin may be an autocrine or paracrine growth factor for both leiomyoma and myometrial cells (Nowak *et al.* 1999). At this date, it would seem that the prolactin story is unfinished, evolving, and worthy of further study.

1.1.2. Initiators of Tumorigenesis:

1.1.2.1. Theories of Initiation:

The most important aspect of the etiology of fibroids--the initiator(s)--remains unknown. Several theories have been advanced. One hypothesis states that increased levels of estrogen and progesterone result in an increased mitotic rate that may contribute to myoma formation by increasing the likelihood of somatic mutations (Rein 2000). Another favors an inherent abnormality in the myometrium of those who develop fibroids, based upon the finding of significantly increased levels of estrogen receptor (ER) in the myometrium of fibroid uteri (Richards and Tiltman 1996). Others have suggested a predisposing genetic factor on the basis of ethnic and familial predilections (Marshall *et al.* 1997; Schwartz *et al.* 2000b).

1.1.2.2. The Genetic Findings:

1.1.2.2.1. Heritability:

A rare inherited disorder known as Reed's Syndrome or multiple leiomyomatosis, is characterized by the appearance of multiple leiomyomas in the skin, uterus, or both. The family histories in these cases suggest an autosomal dominant inheritance with incomplete penetrance (Thyresson and Su 1981). Recent reports of several

families with multiple uterine and cutaneous leiomyomata, and a subset of these with papillary renal cell carcinoma, have independently linked this disorder to a predisposition gene in the region of chromosome 1q42.3-q43 (Alam *et al.* 2001). In follow-up studies of this chromosomal region, mutations were detected only in the fumarate hydratase gene (Tomlinson *et al.* 2002)--a surprising finding, as this enzyme is a component of the essential energy-producing cycle {Tri-Carboxylic Acid} (Rustin *et al.* 1997). Furthermore, the gene appears to act as a classic tumor suppressor in that loss of the wild type allele was observed frequently in the leiomyomata and renal cell cancers (Launonen *et al.* 2001). Although this hereditary syndrome is itself rare, the association with inactivation of the fumarate hydratase gene is of interest, as it is possible that other mechanisms of transcriptional silencing of this gene such as promoter hypermethylation could be involved in the development of sporadic leiomyomas (Kiuru *et al.* 2001).

1.1.2.2.2. Clonality:

There is a general acceptance in most literatures that these leiomyoma tumors are monoclonal. The underlying premise of these studies has been based on the Lyon hypothesis, which assumes that only one X chromosome is active in any female cell, the other X chromosome remaining in an inactive state, and that the X chromosome that is inactivated (methylated) is determined randomly. Thus, genetic loci known to be located on the X chromosome can be studied in these tumors for evidence of homogeneity of expression in

those patients identified as heterozygous for a particular gene in their normal, nontumor tissues (Gordon *et al.* 2003).

Vogelstein *et al.* (1985) in their studies have taken advantage of methylation-sensitive restriction enzymes to discriminate between active and inactive alleles of X-linked genes known to be highly polymorphism. Tumors arising from single cells should contain only one type of inactive (methylated) allele, which will be amplified exclusively following restriction-enzyme digestion of the active (unmethylated) allele, whereas tumors of multicellular origin should contain some cells with one type of inactive allele and other cells with a second type of inactive allele, resulting in the amplification of both alleles following digestion and polymerase chain reaction. This method has been employed for analysis of both the X-linked androgen receptor gene (Mashal *et al.* 1994) and the X-linked phosphoglycerokinase gene (Hashimoto *et al.* 1995). Both studies concluded that the uterine fibroids examined were monoclonal in origin.

1.1.2.2.3. Cytogenetics:

Most of the investigations of leiomyomas that search's for chromosomal aberrations have used classic cytogenetic karyotyping, a valuable tool because it is the only method that allows one to survey the entire genetic constitution of a tissue with a single assay. Standard cytogenetic methodology with G-band analysis can identify translocations, deletions, and duplications, but it does require the *in*

vitro culture of leiomyoma cells to obtain metaphase preparations. A genomic hybridization, which is an alternative comparative method, permits the recognition of cytogenetic changes such as deletions and amplifications without the need for cell cultures of the tumor. Neither standard karyotyping nor comparative genomic hybridization permits the detection of small, submicroscopic chromosomal abnormalities such as point mutations or epigenetic changes such as methylation (Levy *et al.* 2000 ; Packenham *et al.* 1997).

As a result of studying the correlation the tumor phenotype, there was no indication of systematic histological differences between leiomyomas with normal karyotypes and only those with chromosomal aberrations were found in one study (Nilbert and Heim 1990); however, there is some evidence from other reports (Meloni *et al.* 1992; Pandis *et al.* 1990) that leiomyomas that are either cellular with mitotic activity or atypical histologically are more likely to demonstrate karyotypic abnormalities or to show massive karyotypic aberrations indicative of clonal evolution.

In a study of 114 myomas from 92 patients, myomas > 6.5 cm demonstrated a significantly higher proportion of abnormal karyotypes than myomas < 6.5 cm (75% vs. 34%) (Rein *et al.* 1998). In the same study a relationship between particular karyotypes and fibroid size was identified, with the largest tumors carrying abnormalities and the smaller tumors exhibiting chromosome 7 deletions, suggesting that chromosomal abnormalities associated with individual myomas may enhance myoma growth. And a correlation between the location of the fibroid and the likelihood of a cytogenetic abnormality has also been reported; that submucous myomas

presented significantly fewer abnormal karyotypes (12%) than did either the intramural (35%) or the subserosal (29%) tumors, and furthermore, this correlation remained significant regardless of the diameter of the myoma (Brosens *et al.* 1998).

1.1.3. Promoters: Evidence for the Role of Estrogen and Progesterone:

Estrogen has been considered as the primary promoter of uterine leiomyoma growth. This supposition has been based in part upon the clinical observations that fibroids occur only after menarche, develop during the reproductive years, may enlarge during pregnancy, and frequently regress following menopause. Cramer 1992;and Romieu *et al.* 1991 have proposes that unopposed estrogens is the underlying cause of uterine fibroids because the risk of fibroids found greater in the nulliparous women who might be subject to a higher frequency of anovulatory cycles and also the obese women with greater aromatization of androgens to estrone in the fat. Increased growth of myomas among women taking tamoxifen or receiving transdermal or injected estrogen-replacement therapy further supports the importance of estrogen. The estrogen hypothesis has also been supported by clinical trials evaluating the medical treatment of myomas with gonadotropin releasing hormone (GnRH) agonists, the effective result of which is hypoestrogenism accompanied by regression of the fibroids (Friedman *et al.* 1989). As noted by Rein, however, distinguishing the relative importance of estrogen versus progesterone

is difficult, as progesterone levels, in a manner similar to those of estrogen, are also cyclically elevated during the reproductive years, are significantly elevated during pregnancy, and are suppressed after menopause (Rein *et al.* 1995). Furthermore, regression of uterine leiomyomata has been induced by treatment with the anti-progesterone drug (RU 486), accompanied by reduction in the progesterone receptor (PR) but not the estrogen receptor (ER) in the tumors, suggesting that the regression was attained through a direct anti-progesterone effect (Murphy *et al.* 1993). In addition patients treated with leuprolide (a GnRH agonist capable of reducing the size of fibroids) who were concomitantly given medroxyprogesterone acetate demonstrated no significant reduction in myoma or uterine volume (Carr *et al.* 1993).

1.1.4. Effectors: Growth Factors and Their Receptors:

The growth-promoting effects of estrogen and progesterone upon the myometrium and uterine myomas may be mediated through the mitogenic effects of growth factors produced locally by smooth muscle cells and fibroblasts (Mangrulkar *et al.* 1995). Growth factors are polypeptides or proteins that are secreted by a number of cell types, have a wide range of biologic effects, and generally act over short distances either in an autocrine or paracrine manner. They are essential elements in controlling the proliferation rate of cells, and overexpression of either the growth factor or its receptor may contribute to tumorigenesis. Growth factors exert most of their effects on target cells by interaction with specific cell-surface receptors, with subsequent

message transmission via signal transduction systems in the cell. Even in the physiological state, the cellular responses induced by growth factors are complex and dependent upon a number of variables, including the cell type, the differentiation stage of the cell, other stimuli acting simultaneously upon the cell, and the tendency for most growth factor receptors to interact with an entire family of growth factors (Pusztai *et al.* 1993).

1.1.4.1. Growth Factors Identified in Fibroids:

Several growth factors and their receptors have now been identified in both myometrium and leiomyomas, *e.g.* transforming growth factor(TGF)-ß, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) (Gordon *et al.* 2003).

1.2. Prolactin and its receptor:

1.2.1. Prolactin:

Prolactin (PRL) is a polypeptide hormone synthesized and secreted by lactotrophs, which are acidophilic in the anterior pituitary gland. The lactotrophs account for approximately 15-20% of the cell population of the anterior pituitary gland. However, this percentage increases dramatically in response to elevated estrogen levels, particularly during pregnancy. Prolactin levels are higher in females

than in males, and the role of prolactin in male physiology is not completely understood. Plasma concentrations of prolactin are highest during sleep and lowest during the waking hours in humans.

Prolactin and Growth hormone (GH), along with placental lactogens (PLs), form a family of hormones that probably result from the duplication of an ancestral gene. Its structure is similar to that of GH, contains 198 amino acids and has three intracellular disulfide bridges. Its molecular mass is 22000 d.

French researchers were the first to identify a pituitary factor capable of inducing milk secretion in rabbits (Stricker and Grueter, 1928). American scientists made similar observations, and in addition to naming the new pituitary factor prolactin, showed that PRL was able to stimulate the growth of the pigeon crop sac (Riddle *et al.* 1933).

Prolactin regulates its own secretion through a short-loop feedback mechanism by binding to prolactin receptors located in neuroendocrine dopaminergic neurons; this binding leads to increased hypothalamic dopamine synthesis. When the concentration of dopamine in the hypothalamo-hypophysial portal blood rises, the release of PRL from the lactotrophs is suppressed (Molina 2004).

Pituitary lactotrophs have the ability to secrete PRL spontaneously and the secretion is therefore mainly regulated through inhibitory control. Hypothalamic neuroendocrine neurons produce PRL-inhibiting factors (PIF). The primary PIF is dopamine. Additional PIF are somatostatin and γ- amino butyric acid (GABA). PRL regulates its own secretion through negative feedback, and elevated serum PRL increases hypothalamic dopamine synthesis.

Suckling stimuli when nursing pups have been characterized as a neuroendocrine reflex and it increases pituitary PRL secretion. In humans, the baseline serum PRL level was 268 ± 24 ng/ml during lactation and the PRL level was reported to rise to 362 ± 31 ng/ml after 15 minutes of suckling. The control of suckling-induced PRL secretion is still not fully understood. However, suckling stimuli have been demonstrated to reduce dopamine release into portal blood. Additional factors are likely to be involved, for instance vasopressin. PRL secretion is also regulated by environmental factors, such as light, and the release of PRL is higher during sleep compared to the waking hours. Several studies have demonstrated that dopamine levels also change throughout the day. Other environmental factors affecting PRL secretion are stress, olfactory stimuli and sound (Weitzman *et al.* 1980 ; Freeman *et al.* 2000).

In addition to its secretion from the anterior pituitary gland, extrapituitary cells also secrete PRL. Circulating PRL, acting in an endocrine fashion, is mainly secreted from the lactotrophs. The lactotrophic cell population is heterogeneous and the capacity of the cells varies depending on their secretary granule size, content, location and responsiveness to stimuli. In the anterior pituitary gland, there is an additional PRL-producing cell type, the mammosomatotrophs. These cells are bifunctional, and they can produce both PRL and GH.

A number of tissues produce extrapituitary PRL, including brain, lachrymal gland, bone marrow, deciduas, myometrium, skin fibroblasts, spleen, mammary epithelial cells, sweat glands, lymph

nodes and thymus. Extrapituitary-produced PRL often acts in a paracrine and autocrine fashion.

Pituitary and extrapituitary cells use different promoter regions when regulating the expression of PRL mRNA. Pituitary cells use a promoter with three regulatory domains, a proximal region with three Pit-1 binding sites, a distal region with eight Pit-1 binding sites in addition to an estrogen response element (ERE) sequence, and a super distal region. Extrapituitary cells use a promoter with a start site, 5.8 kb upstream of the pituitary start site (Ben-Jonathan *et al.* 1996).

1.2.2. The PRL /GH/PL family:

At the end of the 1970's, the rapid development of cloning technology allowed the identification of the nucleotide sequence of PRL complementary DNAs (cDNAs) from several species. As anticipated from earlier structural studies, the primary structure of PRL appeared closely related to that of two other hormones, growth hormone (GH), also of pituitary origin, and placental lactogen (PL), secreted by mammalian placenta. Today, genetic, structural, binding, and functional studies of these three hormones, as well as the more recently identified somatolactin and PRL-related proteins, have clearly

demonstrated that they all belong to a unique family of proteins (Nicoll *et al.* 1986 ; Goffin *et al.* 1996).

1.2.3. Prolactin tertiary structure:

As a traditional hormone, prolactin is produced by lactotrophic cells in the pituitary and secreted into the bloodstream where it acts distally to regulate reproduction and promote lactation. Pituitary cells store prolactin in secretory granules organized around large prolactin aggregates, which are produced within the trans layer of the Golgi complex. Keeler *et al.* (2003) has determined the tertiary structure of human prolactin using three-dimensional (3D) and four-dimensional (4D) heteronuclear NMR spectroscopy. As expected, prolactin adopts an "up-up-down-down" four-helical bundle topology as can be seen in (Figure1.1).

Prolactin displays three discrete structural differences from growth hormone: ***(1)*** a structured N-terminal loop in contact with the first helix, ***(2)*** a missing minihelix in the loop between the first and second helices, and ***(3)*** a shorter loop between the second and third helices lacking the perpendicular mini-helix observed in growth hormone. Residues necessary for functional binding to the prolactin receptor are clustered on the prolactin surface in a position similar to growth hormone. The tertiary structures of growth hormone and placental lactogen have been determined using X-ray crystallography complexes to the extracellular domains of their functional receptors (Elskins *et al.* 2000; Clackson *et al.* 1998). Nuclear Magnetic

Resonance (NMR) spectroscopy has been used to characterize the tertiary structures and backbone dynamics of many members of this protein family including interleukin-4 (Redfield *et al.* 1992), granulocyte colony-stimulating factor (Zink *et al.* 1994), interleukin-6 (Xu *et al.* 1997), leukemia inhibitory factor (Purvis and Mabbutt 1997), erythropoietin and growth hormone (Cheetham *et al.* 1998).

Figure (1.1): Ribbon model of the three dimensional structure wild-type human prolactin. The highlighted sections are the tryptophan amino acid (Keeler *et al.* 2003).

There are several forms of human prolactin (hPRL) circulating in the blood. Monomeric hPRL is the major circulating hormone. Dimeric hPRL ranges between 8% and 20% while polymeric human PRL (hPRL) is between 1 and 5% of circulating hPRL. Both polymers and dimmers are PRL's form of storage, which occur in storage granules and link up each unit by S-S bond (Figure 1.2). During secretion, most polymeric forms of hPRL are broken to monomers by reduction (Patmastan 2003).

Monomeric form is the major form of PRL circulating in the blood.

Some polymers fail to be completely reduced during secretion. However, polymers can be dissociated by reducing agents or digested with enzymes such as chymotrypsin. The dimeric and polymeric hPRLs have a decreased receptor binding and biological activity.

Rambourg *et al.* (1992) has reported that prolactin forms large aggregates in the lumen of the trans layer of the Golgi complex, which are necessary for the formation of secretory granules.

Prolactin aggregates are differ from many other protein aggregates in that they are readily reversible; when they aggregates, they are released from the cell, they rapidly dissolve, releasing soluble, and correctly folded to produce the monomeric and functionally active protein. Both the mildly acidic pH in the trans-Golgi lumen, and facilitation by binding Zn^{+2} in addition to the high concentrations of prolactin in the secretory pathway form the driving forces for prolactin aggregation in the trans-Golgi lumen (Sankoorikal *et al.* 2002).

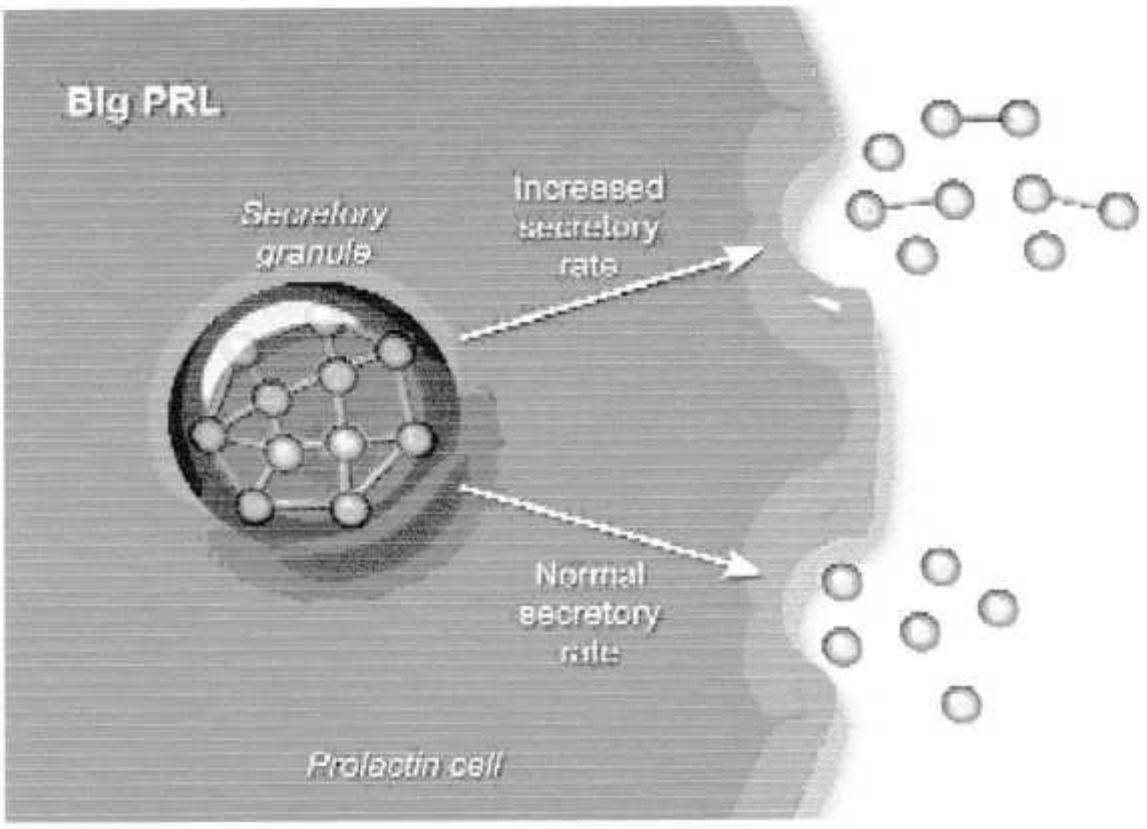


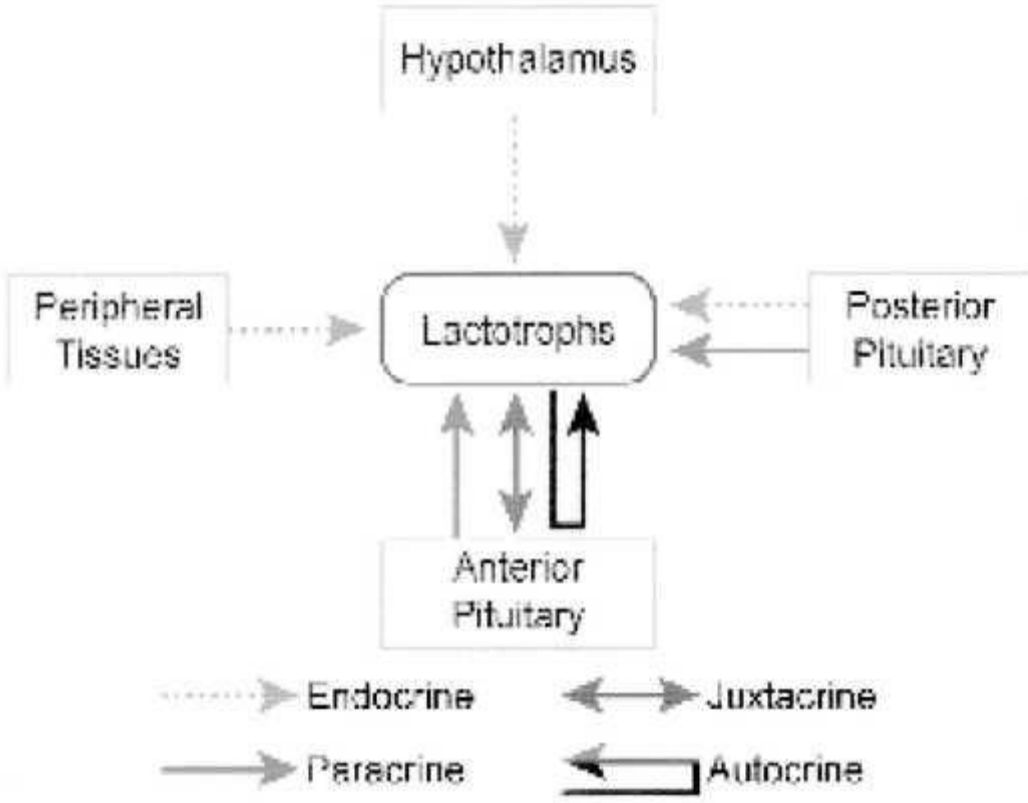
Figure (1.2): Various polymeric forms of hPRL are produced during secretion (Patmastan 2003).

The biochemical nature of the aggregate that exists within the dense cores of secretory granules affects their function. Properly functioning secretory granules require specific membrane proteins to accumulate around the aggregate. However, all aggregates of protein forming in the trans-Golgi lumen do not result in functional secretory granules (Castle *et al.* 1995).

Zhu *et al.* (2002) suggest that a single amino acid change in prolactin or growth hormone results in a change in the retention of the secretory granules that store these proteins.

1.2.4. PRL as an Endocrine, Autocrine and Paracrine Hormone:

Functionally, prolactin has been characterized as both an endocrine hormone and an autocrine/paracrine growth factor (Figure 1.3). As a hormone, prolactin is expressed by lactotrophic cells in the anterior pituitary under the control of a well-characterized, proximal "pituitary-type" promoter, which requires the Pit-1 transcription factor for *trans* activation (Ben-Jonathan *et al.* 2002).



Figure(1.3): Regulation of hPRL secretion. Prolactin is secreted from the lactotroph, which was regulated by several tissues such as hypothalamus, peripheral tissues, posterior pituitary and other cell types in the anterior pituitary gland (Patmastan 2003).

Pituitary PRL acts via a classic endocrine pathway, *i.e.*, it is secreted by a gland, transported by the circulatory system, and acts on target cells at some peripheral sites via specific receptors located on the plasma membrane. The PRL that is produced by many different cell types can act in a more direct fashion, *i.e.*, as a growth factor, neurotransmitter, or immunomodulator, in an autocrine or paracrine manner. Thus, locally produced PRL can act on adjacent cells (paracrine) or on the PRL-secreting cell itself (autocrine) (Bole-Feysot *et al.* 1998).

Newly synthesized protein is concentrated within the Golgi apparatus where large aggregates of prolactin are formed that subsequently become the dense cores of vesicles called secretory granules. Release of granules occurs through Ca^{++}-dependent exocytosis, largely regulated by factors secreted from the hypothalamus.

Circulating prolactin acts distally to regulate reproductive function and promote lactation. Investigation of animal models with genetic deletions of prolactin and its receptor suggests additional biological functions for prolactin including bone turnover, maternal behavior and regulation of carbohydrate and lipid metabolism (Goffin *et al.* 2002).

1.2.5. Extrapituitary Prolactin:

Outside the pituitary, prolactin is synthesized in a variety of tissues including breast, prostate, placenta, uterus, endothelium, immune cells and within the central nervous system. The biology of extrapituitary prolactin is distinct. A supradistal promoter with an

alternative first exon and transcription start site controls expression. The resulting extrapituitary transcript is spliced into the pituitary sequence generating an identical coding region. Outside of the pituitary, prolactin is not stored as an aggregate within secretory granules. These differences in genetics and cell biology suggest an alternative physiological role for extrapituitary prolactin. A multitude of actions have been ascribed, most likely a reflection of the nearly ubiquitous distribution of the prolactin receptor. Research supporting prolactin function as an autocrine/paracrine growth factor in cancers of the breast, prostate and reproductive tract has recently been reviewed (Keeler *et al.* 2003).

As PRL can be synthesized in a variety of tissues, it can thus be found in several fluid compartments in addition to serum, such as cerebrospinal fluid, amniotic fluid, tears, milk, follicular fluid, and sweat. Nagy and Berczi (1991) have explained in their study that neutralization of circulating PRL with anti-PRL antibodies will result in immune dysfunction and death, so they suggesting that extra-pituitary PRL is important and, under some circumstances, can compensate for pituitary PRL.

1.2.6. Biological functions of prolactin:

PRL has a great diversity of action in many species and all of them are very interesting. First, PRL was isolated by its ability to stimulate mammary development and lactation as well as to stimulate the production of crop milk in pigeons. PRL functions in a number of ways, which can be grouped into several categories (Bole-Feysot *et al.* 1998).

1.2.6.1. Water and electrolyte balance:

PRL plays a major role in regulating water and electrolyte balance through the gill and kidney of many fish such that it has been referred to as a fresh water-adapting hormone. Besides clearly reducing Na^+ loss or efflux and water uptake or permeability in the gill, it also increases extra-cellular volume in the kidney as well as enhancing Na^+ re-absorption in the bladder of fish and amphibians. In mammals, PRL has been shown to reduce renal Na^+ and K^+ excretion and to simulate Na± K^+ adenosine triphosphatase (ATPase).

Furthermore, PRL decreases Na^+ and Cl^- in sweat and increases water and salt absorption in all regions of the intestines (Pippard and Baylis 1986; Roberson *et al.* 1986).

1.2.6.2. Growth and development:

In amphibians, PRL is best known for its antimetamorphic effects. In larvae, PRL has been shown to increase the growth of gills and caudal fin and to increase tail length. PRL actions have an effect on cell proliferation and development and one of its major targets is skin. In reptiles and amphibians, PRL promotes molting of the epidermis. It simulates skin melanocyte growth in fishes and mammals and keratinocyte growth in mammals. In birds, PRL induces defeathering and epidermal growth of the incubation patch. PRL induces maturation of the lung and surfactant production and maturation of germ cells (Sage 1970; Yoshimura *et al.* 1989).

1.2.6.3. Endocrinology and metabolism:

PRL has been shown to effect energy metabolism by modulating ATPase activity in monkey brain: Na^{+}-K^{+}-dependent ATPase was stimulated while Mg^{2+} and Ca^{2+}-dependent ATPases were reduced in neural as well as glial cells. PRL also has marked effects on lipid metabolism. In birds, it augments lipoprotein lipase activity in adipocytes although this effect is not seen in mammals, as the adipocyte does not have PRL receptors. In mammals, PRL stimulates phospholipid synthesis in the fetal lung and lipoprotein lipase activity in liver. PRL has been shown to increase bile secretion and to have a direct effect on pancreatic function, increasing insulin secretion (Machida *et al.* 1990; Sorenson *et al.* 1987).

1.2.6.4. Brain and behavior:

PRL has been suggested to be involved in parental behavior of fishes, birds and mammals. In fishes, PRL has been implicated in fin fanning, to provide a constant supply of fresh water to the eggs and to stimulate mucus production, which is used to feed the young after they hatch. Another form of parental behavior is foam nest building in which air bubbles are mixed with mucus to form bubbles during egg laying. PRL is also involved in migration (Blum and Fiedler 1965).

1.2.6.5. Reproduction:

Actions related to the processes of reproduction represent the largest group of different functions identified for PRL. The different actions are quite diversified, such as nurturing of the young. PRL is best known for its actions on the mammary gland: the growth of the

gland that occurs during pregnancy is under the control of a number of trophic factors including estrogen, progesterone, insulin, glucocorticoid, GH, and PRL or placental lactogen (PL). The terminal stage of mammary gland development, lobuloallvelolar growth is directly regulated by PRL. PRL is the hormone primarily responsible for the synthesis of milk proteins, lactose and lipids, all major components of milk. In addition, PRL has been shown to directly stimulate IGF-I binding protein.

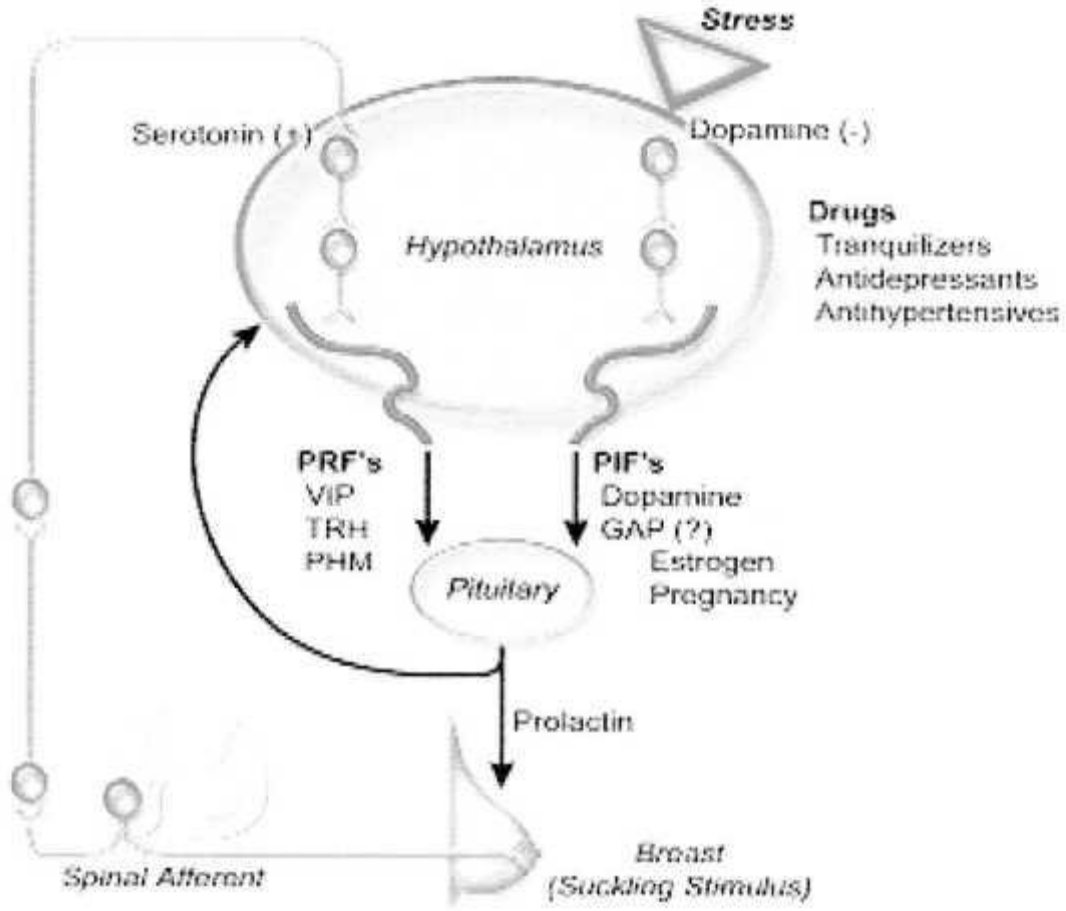


Figure (1.4): Regulatory pathways stimulate or suppress prolactin secretion (Patmastan 2003).

The major prolactin releasing factors (PRF) are thyrotropin releasing hormone (TRH) and vasoactive intestinal polypieptide (VIP). The major prolactin inhibitory factor (PIF) is dopamine. Stress and drugs can influence the inhibition of prolactin secretion. All of the regulatory factors have effects at the hypothalamus (Figure 1.4) (Neville and Daniel 1987).

1.2.6.6. Ovarian actions:

In rodents, the luteotropic action of PRL generally involves stimulation of progesterone production by luteal cells. In mammals, depending on the stage of the cycle, luteolytic effects of PRL have also been reported. Several factors, including PRL, seem to be involved in the destruction of the corpus luteum.

In granulosa cells, PRL inhibits estrogen synthesis and P_{450} aromatase and induces α-2-macroglobulin via activation of Stat5. As a factor regulating the formation and destruction of the corpus luteum, PRL seems to play a major role in modulating the physiological states of estrus, pregnancy and lactation. A direct effect of PRL on developmental competence and maturation of oocytes has been reported in rabbits.

The addition of PRL to the oocyte maturation medium increased the development of organized embryos. PRL was also able to directly inhibit degeneration and decomposition of surface epithelial cells and the disiruption of connective tissue at the apex of the follicle wall.

1.2.6.7. Uterine actions:

In the uterus, PRL is able to increase the level of progesterone receptors and thus all actions associated with this steroid hormone are enhanced.

PRL promotes blastocyst implantation.

1.2.6.8. Testicular actions:

In Leydig cells; PRL increases LH receptor number and increases steroidogenesis and androgen production.

In germ cells, PRL increases the spermatocyte-spermid conversion.

1.2.6.9. Male sex accessory actions:

PRL increases the weight of the prostate and seminal vesicle. In the seminal vesicle, PRL increases lipid concentrations in the fluid. The effects of PRL on prostate include increased levels of androgen receptor (Dombrowicz *et al.* 1992; Nag *et al.* 1981).

1.2.6.10. Immunoregulation and protection:

In lymphocytes, PRL is known to increase antibody formation, including immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies and to induce cellular proliferation. PRL has been shown to inhibit apoptosis of lymphocytes. Administration of PRL is also associated with increased graft rejection and an increase in T-cell engraftment. Macrophage activation and superoxide anion production

responsible for killing pathogenic organisms are effects mediated by the PRLR (Lahat *et al.* 1993).

1.2.6.11. Actions associated with pathological disease states:

In humans, hyperprolactinemia has been shown to be associated with amenorrhea, galactorrhea, and impotence (Clevenger *et al.* 1997). The inhibitory effects on the reproductive processes may be due to both central and peripheral actions of PRL. In some women, elevated PRL is associated with a psychosomatic state of pseudopregnancy (Shewade and Ramaswamy 1995 ; Sobrinho 1993).

Interestingly, however, no genetic diseases associated with a mutation of the gene encoding PRL or the PRLR have been identified in humans or animals. Either these genes are not important or they are essential to the proper survival of the species.

An excessive volume of amniotic fluid, known as polyhydramnios, is associated with decreased levels of amniotic fluid PRL or PRLR levels in the chorion laeve. This effect may be related to the osmoregulatory role of PRL during fetal life and to the inhibitory effect on amniotic fluid volume observed in monkeys (Sarandakou *et al.* 1992; Josimovich *et al.* 1977).

PRL has been associated with a number of different forms of cancer. For example, PRL is thought to increase colorectal tumor agressivity (Bhatavdekar *et al.* 1994), induce the proliferation of several lines of human breast cancer (Das and Vonderhaar 1996),

activate malignant ß-lymphocytes and lymphoma cells, and induces the proliferation of promyelocytes (Walker *et al.* 1995).

Benign fibro muscular myometrial tumors (leiomyomas) have been shown to produce more PRL than control myometrium; thus, locally produced PRL may exert a mitogenic action on the growth of these tumors (Nowak *et al.* 1993).

PRL has been shown to be increased and to affect a number of autoimmune states, such as systemic lupus erythematosus (El-Garf *et al.* 1996), acute experimental allergic encephalomyelitis (Walker *et al.* 1993), rheumatoid arthritis and adjuvant arthritis (McMurray *et al.* 1995). PRL has also been suggested to be involved in the etiology of cystic fibrosis, although the precise mechanism remains unclear (Kulczycki and Robertson 1988).

1.2.7. Hyperprolactinemia and prolactin metabolism:

In human patients with pituitary tumors, prolactinomas are very common. The main signs of hyperprolactinemia in women are amenorrhea and galactorrhea. In addition, chronic hyperprolactinemia leads to metabolic disorders (Cohen *et al.* 1984). Landgraf *et al.* (1977) and Schernthaner *et al.* (1985) have demonstrated that hyperprolactinemia induces an insulin-resistant state in humans. They have improved this when they treated patients with hyperprolactinemia with bromocriptine, and found that this drug will act as a dopamine agonist that suppresses PRL secretion, glucose tolerance and insulin sensitivity.

Reis *et al.* (1997) have demonstrated in hyperprolactinemic rats, both hyperglycaemia and insulin resistance. However, insulin resistance was more pronounced in female rodents with hyperprolactinemia compared to males and estrogen was suggested to affect PRL's diabetogenic effects in females.

Chronic high PRL levels have been suggested to increase the body weight in human patients. However no difference in body weight was observed in patients with microprolactinoma (Greenman *et al.* 1998; Freemark *et al.* 2001).

In rodents, the effect of PRL on adipose tissue size has been contradictory, but elevated PRL levels would increase food intake and body weight in rodents (Matsuda *et al.* 1996).

Freemark *et al.* (2002) have reported that PRL has the effect to stimulate insulin secretion by increasing the proliferation of pancreatic islet β-cells. In addition, PRLR-deficient, PRLR-/- mice have reduced pancreatic islet density, β-cell mass and insulin content as well as blunted insulin secretion and impaired glucose tolerance in response to glucose administration. *In vitro*, PRL has been demonstrated to reduce insulin binding to human adipocytes from women at term gestation and decrease glucose transport in rat adipocytes (Jarrett *et al.* 1984; Ryan and Enns 1988).

1.2.8. The Macroprolactin Problem:

Human prolactin (PRL) is synthesized as a pre-hormone with a molecular weight of 26 kDa. When the pre-prolactin is cleaved, the resulting polypeptide has a molecular weight of 23 kDa, and this monomeric form accounts for the majority of total PRL (Schlechte

2002). Prolactin (PRL) in human serum has been classified into three main species on the basis of molecular mass: monomeric PRL, big PRL and big, big PRL (bb-PRL), called 'macroprolactin', with molecular masses of 23 kDa, 50–60 kDa and 150–170 kDa respectively (Smith & Norman 1990). Although the nature of bb-PRL is heterogeneous, the most common form of macroprolactin is a complex of PRL and immunoglobulin G (Lindstedt 1994; De Schepper *et al.* 2003). Most patients with macroprolactinemia do not show any clinical symptoms of hyperprolactinemia, such as amenorrhea, galactorrhea and infertility, despite high hyperprolactinemic levels and they do not need specific treatment (Leslie *et al.* 2001).

Macroprolactin is recognized, in various degrees, by immunoassays for PRL and has a slower clearance from serum than PRL, causing diagnostic confusion in evaluating hyperprolactinemic conditions. The incidence of macroprolactinemia ranges from 15% up to 26% of all hyperprolactinemic sera and represents the main cause of inter assay variability for PRL dosage (Cavaco *et al.* 1999; Smith *et al.* 2002).

In general, big big PRL consists of an antigen antibody complex of monomeric PRL and IgG. When the serum of a patient with hyperprolactinemia contains mostly big big PRL the condition is termed *macroprolactinemia*. Macroprolactinemia has been recognized for many years and is suspected when a patient with hyperprolactinemia lacks typical symptoms and/or has no radiographic evidence of a pituitary tumor (Hattori and Inagaki 1997; Whittaker *et al.* 1981).

Macroprolactinemia is seen in both sexes and in children. And the increase in big big PRL that occur during pregnancy due to binding of PRL to specific autoantibodies. The incidence of macroprolactinemia in patients with hyperprolactinemia ranges from 18–42% when samples from reference laboratories are assayed. In contrast, the incidence of macroprolactinemia in patients from an endocrinology practice is closer to 10% (Vallette-Kasic *et al.* 2002).

If big big PRL is biologically active, the effects may be blunted because of decreased its bioavailability. The large PRL-Ig complex may fail to reach receptors because of limited capacity to cross-vascular endothelium. Although many patients with macroprolactinemia lack typical symptoms of an elevated PRL, there are multiple reports of patients with macroprolactinemia who present with amenorrhea, galactorrhea, and infertility. In a prospective analysis, roughly one third of women with macroprolactinemia had amenorrhea and infertility (Olukoga and Kane 1999; Vallette-Kasic *et al.* 2002). Hauache *et al.* (*2002*) *showed* that the presence of macroprolactin does not totally exclude the possibility of a pituitary adenoma, but asymptomatic patients with macroprolactin usually have normal radiographic studies.

Recognizing the presence of macroprolactin may help define the etiology in patients with idiopathic hyperprolactinemia, and in some cases recognition of macroprolactinemia might eliminate the need for extensive diagnostic tests or pituitary imaging. This is especially important because 10% of healthy subjects have radiographic evidence of a pituitary adenoma (Aron and Howlett 2000).

Smith *et al.* (2002), emphasize the difficulty of detecting macroprolactin with commonly used PRL immunoassays. They sent sera from patients with macroprolactinemia to l8 clinical laboratories in the United States and Europe. In nine assay systems, the difference in PRL varied from 2.3- to 7.8-fold, and in each case PRL measured after removal of macroprolactin was consistently lower than PRL reported by the immunoanalyzer.

Schneider *et al.* (2001); also noted that PRL assays vary remarkably in reactivity for macroprolactin. These and other studies suggest that there is no single PRL assay that will give a normal level of monomeric PRL in the presence of big big PRL, but that some assays are better than others. It is surprising that the results from different assays are so discrepant. It is possible that the differences in cross-reactivity are due to the nature of the macroprolactin autoantibody complex, which may mask epitopes that are recognized by the antibodies in the assay (Olukoga and Kane 1999).

There is no simple method for detection of big big PRL, and clinicians may not be aware that many commercial assays do not provide a procedure for detection of macroprolactin. Gel filtration chromatography is time consuming, expensive, and not used in clinical laboratories. Polyethylene glycol (PEG) precipitates macroprolactin, leaving reduced levels in the supernatant. PEG precipitation is a relatively simple and inexpensive technique but is not specific or quantitative. A percentage recovery of greater than 65% confirms the presence of monomeric PRL whereas a percentage recovery of 40% or less is very sensitive for detecting significant amounts of macroprolactin. Recovery between 40% and 65%

indicates a sample may contain macroprolactin and oligomeric PRL, in addition to the monomeric form. In these cases, separation method such as gel filtration chromatography or electrophoresis would be necessary to confirm the presence of macroprolactinemia (Olukoga and Kane 1999; Leslie *et al.* 2001).

The presence of macroprolactinemia in a patient with no clinical suspicion of hyperprolactinemia could obviate the need for a pituitary magnetic resonance imaging or other testing (Cavaco *et al.* 1999).

Smith *et al.* (2002); also noted that some patients with macroprolactinemia have elevated levels of monomeric PRL and suggest that the diagnosis of macroprolactin be used only when a PRL level falls to a level seen in sera from normoprolactinemic subjects treated with PEG. Although this would help ascertain whether an excess of monomeric PRL is present along with macroprolactin, it would require establishment of new reference ranges for all PRL assays.

1.2.9. The prolactin receptor (PRLR):

The PRLR belongs to the class 1-cytokine receptor family and it was first identified during the seventies. Class 1-cytokine receptors are single-pass transmembrane proteins that contain an extracellular, a transmembrane and an intracellular domain (Ling 2002).

When ligands binding to the extracellular domain, the PRLRs will homodimerise, resulting in activation of intracellular signaling systems.

The extracellular region of the PRLR is 210 amino acids (aa) and contains two subdomains, D1 and D2 (Bole-Feysot *et al.* 1998).

These extracellular subdomains are structured into two types 3 fibronectin-like domains that consist of seven antiparallel β-strands divided into two β-sheets that are connected by a 5 amino acids linker (Clevenger and Kline 2001). Furthermore, D1 has two pairs of disulphide-linked cysteins and D2 contains the 'WS-motif' (Trp-Ser-x-Trp-Ser). Rozakis-Adcock and Kelly (1991) have reported that any mutations in the WS-motif or the cysteins lead to impaired receptor function. Two extracellular tryptophans, Trp72 and Trp139, are reported to be involved in ligand binding. There are three glycosylation sites in the extracellular domain of the PRLR and glycosylation of these sites are necessary for directed cell surface targeting (Buteau *et al.* 1998). The transmembrane domain is a 24 aa hydrophobic domain. There are several PRLR isoforms; long, intermediate and short forms, with different intracellular domains. Two intracellular regions within the PRLR are relatively conserved, box 1 and box 2. These regions have been suggested to be important for PRLR signal transduction. Box 1 is a proline-rich hydrophobic motif with a Pro-x-Pro sequence. The short PRLR isoforms lack box 2 (Bole-Feysot *et al.* 1998).

PRL has a multitude of actions in many different tissues and cells. PRL's function varies depending on, for instance, the reproductive stage of the animal. One explanation for PRL's ability to perform this large number of biological actions in different tissues could be the expression of different PRLR isoforms, which is due to alternative splicing. In both mice and humans, four different transmembrane PRLR isoforms have been identified (Bole-Feysot *et al.* 1998).

Davis and Linzer (1989) have reported that the mouse PRLR consists of one long (L-PRLR) and three short forms (S1-, S2-, S3-PRLR). However, in man, a long (L-), an intermediate (I-) and two short forms (S1a- and S1b-) have been identified by Boutin *et al.* (1989) and Hu *et al.* (2001) (Figure 1.5).

Kline and Clevenger (2001); have identified in milk of a number of species, a soluble PRLR correspond to the binding domain of the receptor. In human serum, the soluble PRLR, functioning as a PRL binding protein, was demonstrated at 14 ng/ml, and it form approximately one-third of serum PRL. However, the physiological role of soluble PRLRs *in vivo* remains unsolved.

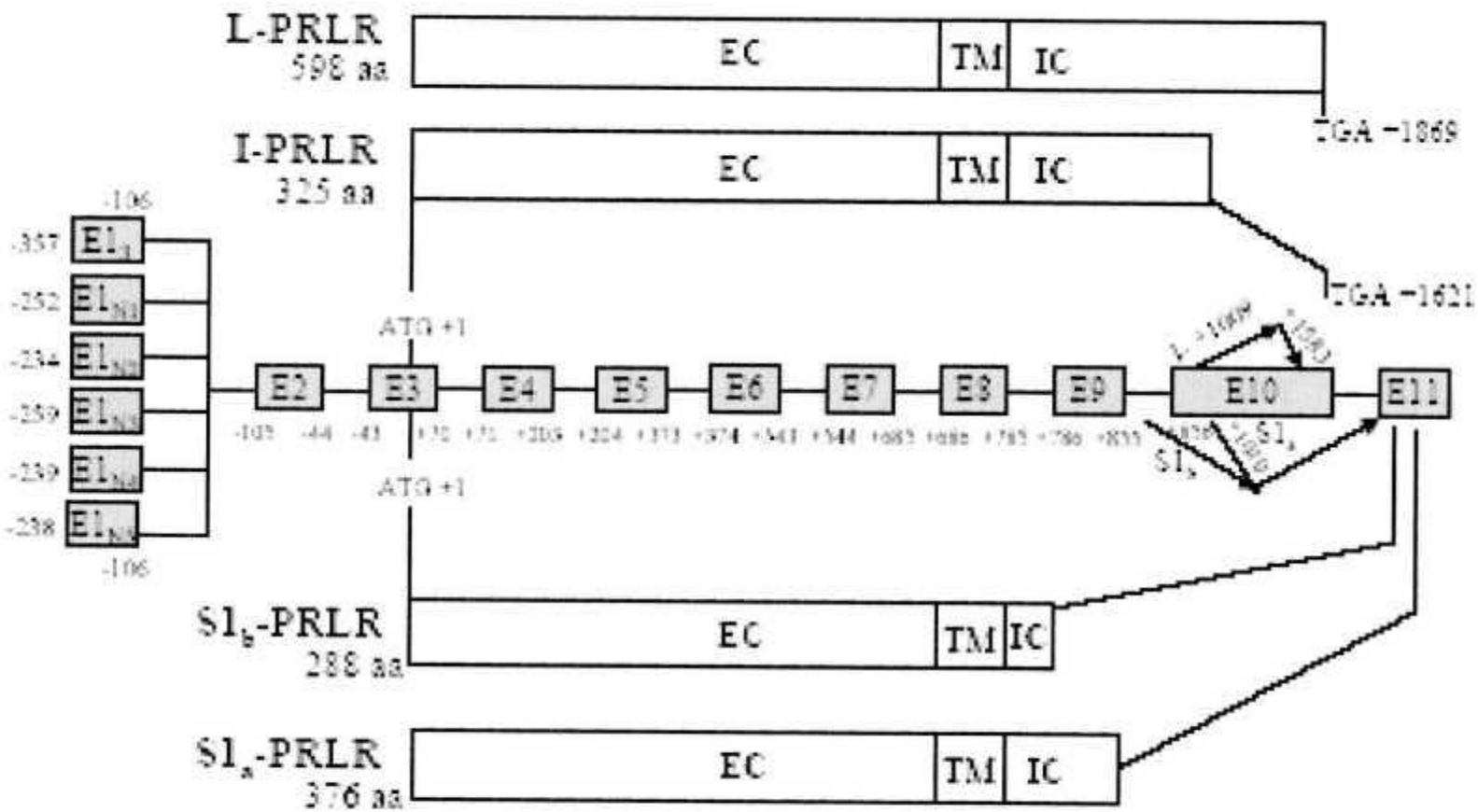


Figure (1.5): The human prolactin receptor gene and the four human prolactin receptor isoforms, L-, I-, S1a- a1nd S1b-PRLR. The human PRLR gene consists of 11 exons (E1-E11). Six alternative E1's have been identified. E1, E2 and part of E3 correspond to the 5'-UTR (untranslated region). . E3-E7 corresponds to the extracellular (EC) region. E8 corresponds to the transmembrane (TM) region. E9-E11 corresponds to the intracellular (IC) regions of the four different PRLR isoforms. This figure is a modified version of (Hu *et al. 1999; 2001; 2002).*

1.2.10. Distribution and regulation of the PRLR:

The PRLR is expressed in a large number of cell types and tissues. They are widely distributed in many vertebrate tissues. They are located in the central nervous system, kidney, bladder (fish, reptiles, amphibians), lymphoid tissues, pituitary, the reproductive system, the skin, bone tissues, gills (fish and larval amphibians), lung, heart, skeletal muscle, adipocytes (birds) and liver.

Prolactin receptors are found not only in plasma membrane but also in endosome Golgi fraction. In rat liver, or lactating mammary gland, most of the receptors are localized in intracellular membranes probably because of rapid receptor synthesis and degradation. Also, in mammary glands, the number of PRL receptors increases markedly in early lactation (Bole-Feysot *et al.* 1998).

The regulation of PRLR expression has been demonstrated to be under hormonal and physiological control in a number of tissues. For instance, PRL was found to increase the number of PRLRs in rat liver (Posner *et al.* 1975). However, Baxter *et al.* (1984) suggested that GH, but not PRL, causes induction of both GHR and PRLR in the rat liver. Moreover, Marshall *et al.* (1978) has demonstrated in their study that estrogen and testosterone are also involved in the regulation of PRLR expression in the liver.

At parturition, serum progesterone decreases and PRL increases. Djiane and Durand (1977) showed that at parturition, progesterone inhibits and PRL stimulates PRLR expression in the mammary gland. They also found a reduction in mammary gland PRLRs when milk production was reduced. Hayden *et al.* (1979) confirmed these results. They found that in the mammary gland, the number of PRLRs was

low during pregnancy, increased during lactation and declined after the litter was weaned. In contrast, in the liver PRL binding increased during pregnancy and decreased during lactation. Jahn *et al.* (1991) has analyzed the Regulation of the long and short PRLR forms in rat mammary gland and liver, and they found that the short form increased predominantly in the liver during pregnancy. In contrast, both forms increased in the mammary gland during lactation. When pregnant rats were treated with the progesterone antagonist (RU 486), PRLR mRNA expression increased in the mammary gland but not in the liver.

In the ovary of rodents, the level of PRLR expression is regulated during pregnancy by follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) (Clarke and Linzer 1993). In addition, the PRLR expression increased in the pancreas during pregnancy (Moldrup *et al.* 1993). Thyroid hormone was found to regulate the level of PRLRs in a number of tissues, including liver, kidney, testis and prostate of male rats (Tiong *et al.* 1992). Furthermore, in rat prostate, it was found that PRL, testosterone and estrogen regulated PRLR expression (Nevalainen *et al.* 1996).

1.2.11. Prolactin signal transduction:

The different functions of PRL in multitudes of cells and tissues have been related to the expression and regulation of different PRLR isoforms as well as the utilization of distinct signaling pathways. The PRL molecule contains two receptor-binding sites, and when exerting its effects, PRL's binding site 1 interacts first with one PRLR,

resulting in a PRL-PRLR complex (Figure 1.6). Thereafter, PRL's binding site 2 interacts with a second PRLR, resulting in PRLR dimerisation and activation (Freeman *et al.* 2000; Bole-Feysot *et al.* 1998).

In addition to other members of the cytokine receptor family, PRLRs lack enzymatic activity and they are therefore associated with a number of kinases. The family of Janus kinases (JAK), JAK1, JAK2, JAK3 and TYK2, are involved in cytokine receptor activation. JAK2 is the primary PRLR-associated Janus kinase and it is constitutively associated with the PRLR at box 1 in the intracellular part of the receptor. Box 1 is rich in proline residues and it is believed to form a SH3 (Src homology region 3) binding domain. However, because JAK2 lacks an SH3-domain, the mechanism for how JAK2 is associated with the PRLR still needs to be evaluated. After ligand stimulation of the receptor, JAK2 activation occurs within 1 minute. It was suggested that ligand-induced receptor dimerisation brings two receptor associated JAK molecules close together, resulting in JAK activation by transphosphorylation of JAKs tyrosines. Activated JAK2 then phosphorylates tyrosine residues on different target proteins, *e.g.* the PRLR. All PRLR isoforms can activate JAK2. However, only tyrosine residues of the long and intermediate PRLR isoforms are phosphorylated after JAK2 activation (Clevenger and Kline 2001). These phosphotyrosines are important in intracellular signaling, because they are potential binding sites for transducer molecules containing SH2 (Src homology region 2) domains. Signal transducer and activator of transcription (Stat) proteins contain a SH2 domain, as well as a DNA binding domain and a C-terminal transactivating

domain. There are seven members in the Stat family, and Stat5a and Stat5b were initially identified as PRL induced mammary gland transcription factor. The SH2 domain of Stat5 interacts with active PRLR-phosphotyrosines, resulting in a PRLR/JAK2/Stat5 complex where JAK2 can phosphorylate Stat5. Two phosphorylated Stat molecules then dimerise through SH2- phosphotyrosine interactions and Stat-dimers translocate to the cell nucleus, where they activate γ-interferon-activated sequence (GAS) motifs in promoters of PRL target genes (Wakao *et al.* 1994; Liu *et al.* 1995).

Active Stat-dimers can operate in combination with other transcription factors to regulate the specificity of transcription (Bole-Feysot *et al.* 1998; Goffin *et al.* 2002). PRL can activate other signaling pathways than the PRLR/JAK2/Stat5 pathway. For example, PRLRs utilize the Src family of protein tyrosine kinases, including Fyn, which is constitutively associated with PRLRs (Clevenger and Kline 2001). The exact role of Src proteins in PRLR signaling remains unclear, but Kline *et al.* (1999) demonstrated that the human L-PRLR but not I-PRLR activates Fyn after PRL stimulation.

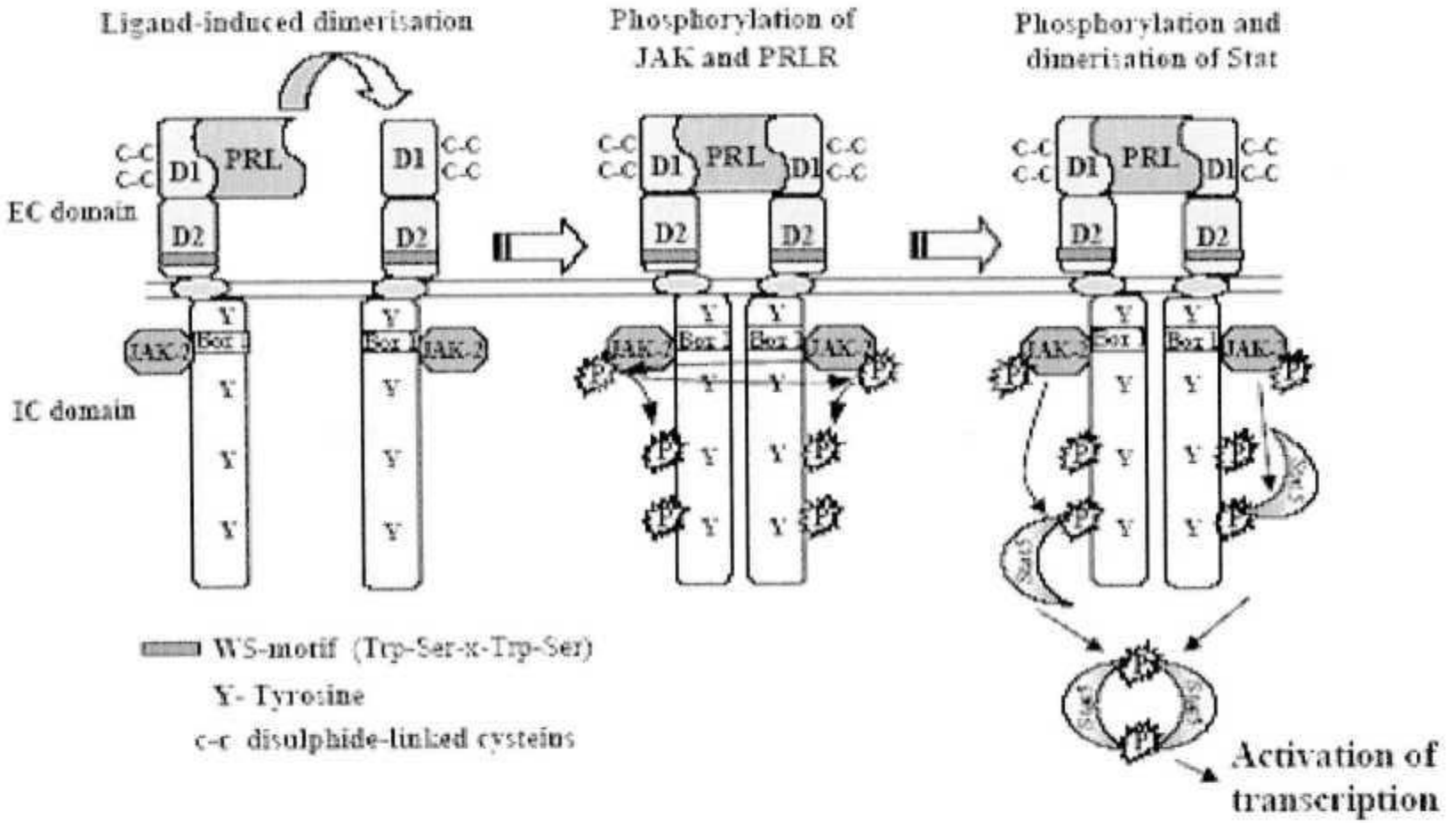


Figure (1.6): Illustration of the prolactin receptor (not drawn to scale) and prolactin-induced signal transduction. The prolactin receptor contains an extracellular (EC) domain, which consists of two subdomains, D1 and D2, a transmembrane domain, and an intracellular (IC) domain. This figure is a modified version from Freeman et al (Freeman *et al.* 2000).

Furthermore, the PRLR forms, together with the tyrosine kinase Tec and the guanine nucleotide exchange factor Vav1, a Tec/Vav/PRLR complex, with suggested roles in cell proliferation (Kline *et al.* 2001). Tec is also involved in activation of the transcription factor c-fos. In addition, suppressors of cytokine signaling 1 (SOCS-1) were found to bind to Tec and down-regulate Tec's kinase activity. The PRLR can also activate the mitogen-activated protein (MAP) kinase pathway, where PRLR-phosphotyrosines interact with adapter proteins like Shc/Grb2/SOS, which connect the PRLR with the Ras/Raf/MAP kinase pathway. Active MAPK is translocated to the nucleus, where transcriptions factors including Myc, Jun and TCF are activated by phosphorylation (Clevenger and Kline 2001). PRL has also been found to activate phosphoinositol-3-kinase (PI3K) and insulin receptor substrate 1 (IRS-1), with possible implications for PRL in insulin signaling and glucose metabolism (Berlanga *et al.* 1997).

1.2.12. Immunohistochemical localization of prolactin receptor:

The prolactin receptor (PRLR) is a transmembrane protein that belongs to the cytokine receptor super family, and is expressed in a wide variety of tissues in addition to the breast (Hennighausen *et al.* 1997). In the 1970s, evidence for the presence of PRLR in human breast carcinoma was presented using biochemical binding assays (Partridge *et al.* 1979). Subsequently, similar assays were used to

study the prognostic relevance of these receptors in breast carcinoma (Bonneterre *et al.* 1987; De Placido *et al.* 1990).

The prolactin receptor was cloned in 1988 by Boutin *et al.* In 1993, Banerjee *et al* characterized the antibody B6.2, a monoclonal antibody directed against the extracellular domain of the human PRLR. This was raised against a membrane enriched fraction from metastatic human breast cancer cells. This antibody has been used successfully for the demonstration of PRLR in gastrointestinal and breast tissue, and non-Hodgkin's lymphoma cell lines. Gill *et al.* (2001) have used B6.2 to investigate the expression of PRLR in a wide variety of normal, benign, and malignant breast tissue sections, to explore the possible presence of alterations that could be useful in studying the biology of breast cancer. They suggested that because prolactin plays an important role in the proliferation and differentiation of normal breast epithelium, so the higher expression of PRLR noted in the cells of most benign and malignant breast lesions, compared with normal cells, could be an important factor in the pathogenesis of these diseases, rather than a reflection of the high proliferate activity of the abnormal cells.

Kloehn *et al.* (2001) have expressed PRLR mRNA at a higher level in fibrotic and cirrhotic liver specimens. In normal tissue, immunohistochemical staining showed a high concentration of PRLR around the central vein and in the epithelium of the bile ducts. This pattern of distribution was lost in fibrosis and cirrhosis. An accumulation of PRLR was demonstrated within the damaged cells. Neither PRL nor PRL mRNA was detectable in normal, fibrotic, or cirrhotic liver. They conclude that PRLR is distributed in normal rat

liver in a typical pattern which is lost with increasing fibrosis. PRL is not produced by rat liver, indicating that PRL does not act through autocrine or paracrine mechanisms.

Dalrymple and Jabbour (2000) have investigated the expression and signaling pathway of PRL and its receptor in the non-pregnant uterus of the common marmoset monkey. They used techniques, in situ hybridization and immunohistochenmistry to localize expression of the PRLR to the glandular epithelium of the endometrium. They concluded that the genes expression of PRL and its receptor is minimal during the proliferative and early secretory phases. However, both PRL and PRL receptor gene expression are up-regulated during the mid to late secretory phase of the ovulatory cycle. The function of PRL in the marmoset uterus is partly linked to the JAK/STAT signal transduction pathway.

Dixon *et al.* (2000) have studied the immunolocalization of [transforming growth factor alpha (TGF-α) , epidermal growth factor (EGF) , insulin like growth factor (IGF) -I, vascular endothelial growth factor ($VEGF_{165,189,121}$) , basic fibroblast growth factor (FGF) -2, EGF receptor (R) , IGF-IRß, and FGFR-1] in uterine leiomyomas and matched myometrial samples taken from seven women (42-47 years of age) in the proliferative phase of the menstrual cycle. Immunolocalization of growth factor peptides was accomplished with either monoclonal or polyclonal antibodies to the amino or carboxy terminus of growth factor peptides or their respective receptors, or against full-length recombinant growth factor. All reactions were conducted using the avidin-biotin complex method. Immunolocalization of TGF-α, EGF, EGF-R, IGF-I, IGF-IRß, FGF-2,

FGFR-1, and VEGF was observed in the cytoplasm of smooth-muscle cells of leiomyomas and matched myometrium. The cytoplasm of vascular smooth-muscle cells expressed TGF-α, EGF, EGF-R, IGF-I, IGF-IRß, FGF-2, FGFR-1, and VEGF, whereas the vascular endothelium was positive for TGF-α, EGF, EGF-R, FGF-2, and FGFR-1 in both leiomyomas and matched myometria. Fibroblasts within the fibrous component of some leiomyomas were positive for IGF-I and FGF-2 and minimally positive for FGFR-1. In addition, the extracellular matrix of leiomyomas showed focal localization of FGF-2 and IGF-I in some tumors. When scores of intensity and percent positive staining were compared, IGF-IRß was significantly increased in the leiomyomas compared to match myometria, whereas EGF was significantly decreased in the uterine leiomyomas compared to matched myometria. They propose that different growth factors, growth factor receptors, and signaling pathways are coordinately turned on and off throughout the menstrual cycle and throughout the life span of the tumors, and that one growth factor/receptor pathway alone is not solely responsible for the growth of these tumors. This has been shown with IGF-I and myometrial smooth-muscle cells, in that IGF-I alone is a weak mitogen for uterine smooth-muscle cells; however, in combination with EGF and PDGF-BB there is significant smooth muscle cell growth (Tang *et al.* 1994).

Chapter Two

Subjects, Materials And Methods

2. Subjects, Materials and methods:

2.1. Subjects:

Sixty-two women patients were collected from 4 hospitals (Al-Khadimyia Teaching Hospital, Al-Noor, Al-Kharch and Al-Saadoon Hospital- Baghdad).

Those patients were diagnosed previously by their physicians as patients with uterine fibroids after proper physical and gynecological examination and confirmed by ultrasound findings. They were prepared for laparatomy either for Total Abdominal Hysterectomy or Myomectomy.

Nine out of 62 uterine leiomyoma patients (14.52%) were excluded from this study. One of them had breast cancer and underwent a chemotherapy treatment; another had thyroidectomy, 6 with adenomyosis and one with micropituitary adenoma.

Also, a myometrium sample was obtained from non-uterine leiomyoma patients (40 pregnant women underwent cesarean as a healthy control to be compared with the myometrium of the same uterine leiomyoma patient. No blood sample was taken from those pregnant women because their serum PRL level is already higher 10 folds than the normal level.

2.2. Chemicals:

All laboratory chemicals and reagents used in this study were of Analar grade unless otherwise specified and were obtained from BDH limited Pool, U.K.

2.3. Instruments:

The instruments used during this study were:

- Spectrophotometer [CE1011; UV and visible; Instruments Cambridge, England].
- PH meter [METTLER TOLEDO; MP220; pH meter; England].
- Micro centrifuge [Micro Centaur; MSE; United Kingdom]
- Cooling centrifuge [Biofuge 17S Heraeus; SEPATECH; Germany].
- Homogenizer [KINEMATICA Gmbh; Scientific Technical Supplies; D-6072 Dreieich- Germany].
- MSE MINOR 35 Centrifuge; United Kingdom.
- VORTEX-GENIE TM [Scientific industries, INC. Bohemia, NY, 11716 USA].
- Sartorius balance [SARTORIUS AGGOTTINGEN BL210S; Germany].
- Electrophoresis system [EISCO; H.T. Power Supply; Made In India].
- Digital Compact Camera [OLYMPUS; CAMGDIA C-60 Zoom; Made in China].
- Mini VIDAS software version VICPTC-R310.051 [bioMérieux / France].

2.4. Buffers:

A stock [Tris (hydroxymethyl) aminoethane] buffer solution (0.1 M) was prepared by dissolving 12.114 gm/L of the salt ($C_4H_{11}NO_3$, Mwt. 121.14) in distilled water.

A (0.02 M) Tris buffer with pH 7.4 was prepared from this stock solution using a (0.1 N) HCL for adjustment of the pH (Dawson *et al.* 1978).

2.5. Serum Collection:

Ten milliliters of venous blood were aspirated from LM patients just before operation, left to clot, and then centrifuged. Part of it was used for measuring the serum PRL level at the same day of operation by using the Prolactin Kit (Biomérieux); [measurement range of the VIDAS PRL kit is 0.5-200 ng/ml]. The range of expected values for the normal menstruating women is (5-35 ng/ml).

Prolactin level was considered normal up to 35 ng/ml. Patients after operation with abnormal serum PRL level (>35 ng/ml) were sent to the Magnetic Resonance Imaging (MRI) or Computerized Tomography (CT) to examine their pituitary .

All serum samples were treated with polyethylene glycol (PEG) to precipitate the big big PRL (high molecular weight PRL) if present. The precipitating method was carried out within 15 days of collection.

Disk gel electrophoresis method was used to determine the molecular weight of serum PRL.

Biuret method was used to estimate total protein in sera.

2.6. Tissue collection:

Uterine Fibroids (leiomyomas) introduced in this study were identified grossly at surgery and confirmed by histological examination to be fibromatous leiomyomatous tissue.

Uterine leiomyoma were immediately immersed in ice-cold saline solution (0.9% NaCl) after recording their dimensions, types, and localized their position in the uterus.

Leiomyomas were dissected free from surrounding myometrium. When two or more leiomyomas were present in the same uterus, samples from several of them were pooled and diced (Daly *et al.* 1984).

A myometrium sample from the same leiomyoma patient also was taken. Also these myometrium tissue samples were immersed immediately in cold saline as described for leiomyoma samples.

Tissue samples were kept at –20°C before processing up to 2 weeks. Then they were weighed, and sliced with scalpel in Petri dish standing on ice. Slices were thawed and minced with scissors, then homogenized with (0.02) M Tris buffer pH 7.4 with a ratio of 1:3 (w:v) tissue to buffer solution using a mechanical homogenizer (Saif Allah 2000).

The homogenate then was filtered through ten layers of nylon gauze, and centrifuged in cooled centrifuge at 4°C in order to precipitate the remaining intact cells and the intact nuclei at 4000 xg for 30 minutes (Rao 1986), where xg express the relative centrifuged force by applying the following relationship (Burtis and Ashwood 1999):

$$RCF = 1.118 \times 10^{-5} \times r \times (rpm)^2$$

Where **RCF** : relative centrifuged force (xg); **r** : radius (cm); **rpm** : round per minute

Tissue supernatant of leiomyoma(s) or myometrium was then used to estimate the PRL level, total protein, treated with polyethylene glycol and determine the molecular weight as done for serum.

Figure (2.1) shows the processing steps done for both serum and tissue samples in patient group.

Figure (2.2) shows the processing steps done for the control myometrium.

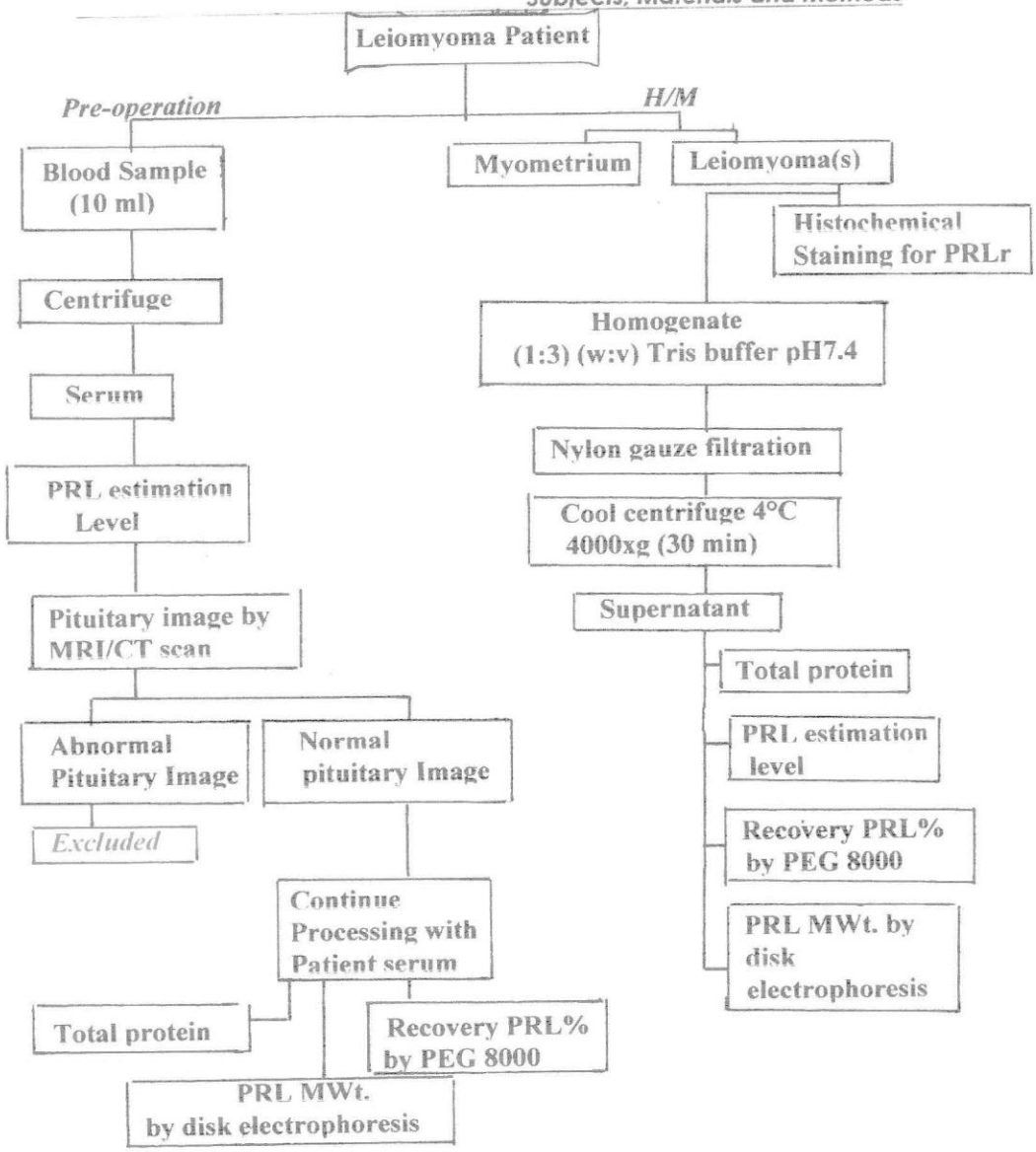


Figure (2.1): processing steps done for both serum and tissue samples in patient group.

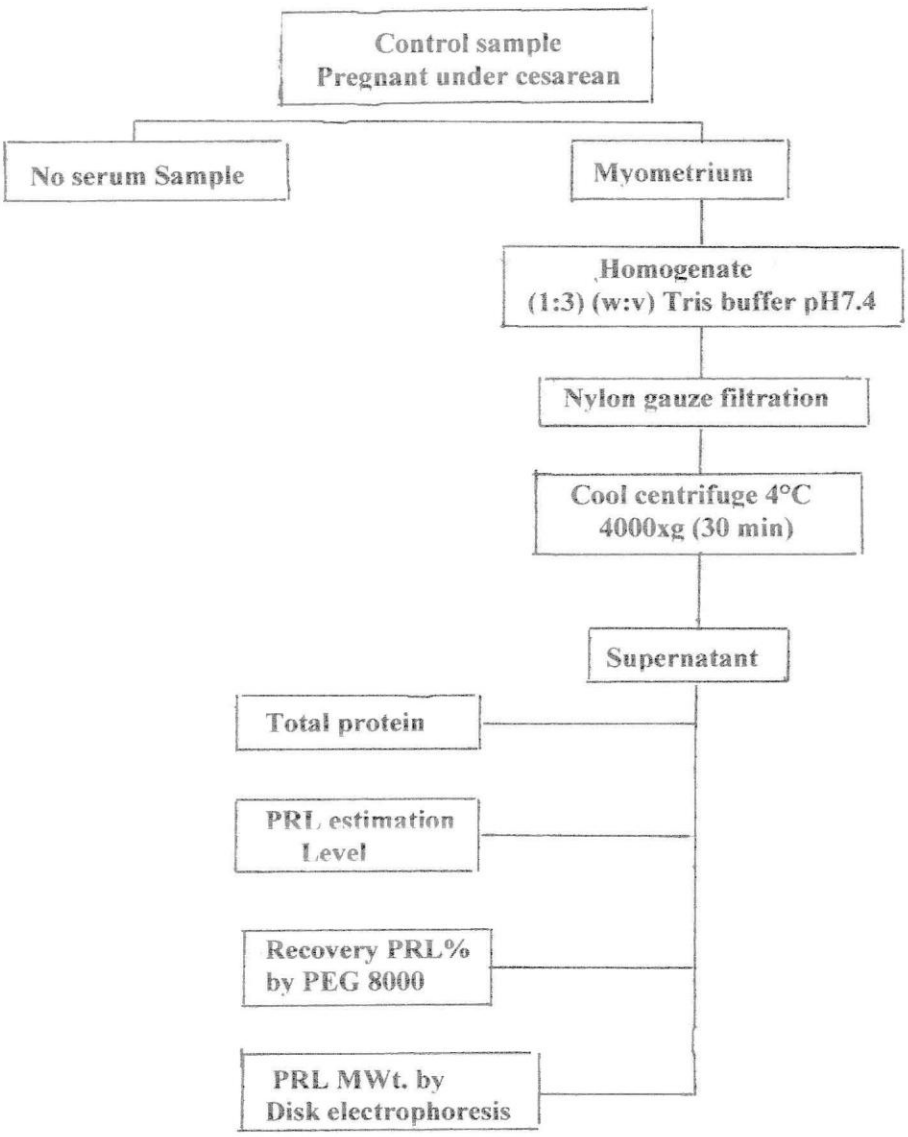


Figure (2.2): Processing steps done for tissue sample in control group.

2.7. Macroprolactin screening method:

All serum and tissue supernatant samples were processed as follows:

The high concentration of polyethylene glycol (PEG) precipitate macroprolactin (big big) from serum, the precipitation method was carried out by adding 200 µl of serum or tissue homogenate supernatant to 200 µl of 250 gm/L PEG 8000 solution (in distilled water, kept at 4°C), mixed for 1 minute with a vortex mixer. The mixture was centrifuged for 5 minutes at room temperature at 9500 xg and again the amount of PRL in the supernatant was determined using the same miniVIDAS. The PRL recovery was calculated according to the following formula (Cattaneo *et al.*2001):

$$\frac{\text{PRL level after PEG precipitation}}{\text{PRL level before PEG precipitation}} \text{ X100}$$

Recoveries ≥65% are classified as predominantly monomeric and recoveries of ≤40% as predominantly high molecular weight forms (macroprolactin). Values between 40-65% were classified as indeterminate and they were all submitted to gel electrophoresis.

2.8. Determination of prolactin molecular weight:

A method of disk gel electrophoresis in sodium dodecyl sulphate (SDS) was used to determine the molecular weight of prolactin in both serum and tissue homogenate supernatant (Figure 2..3).

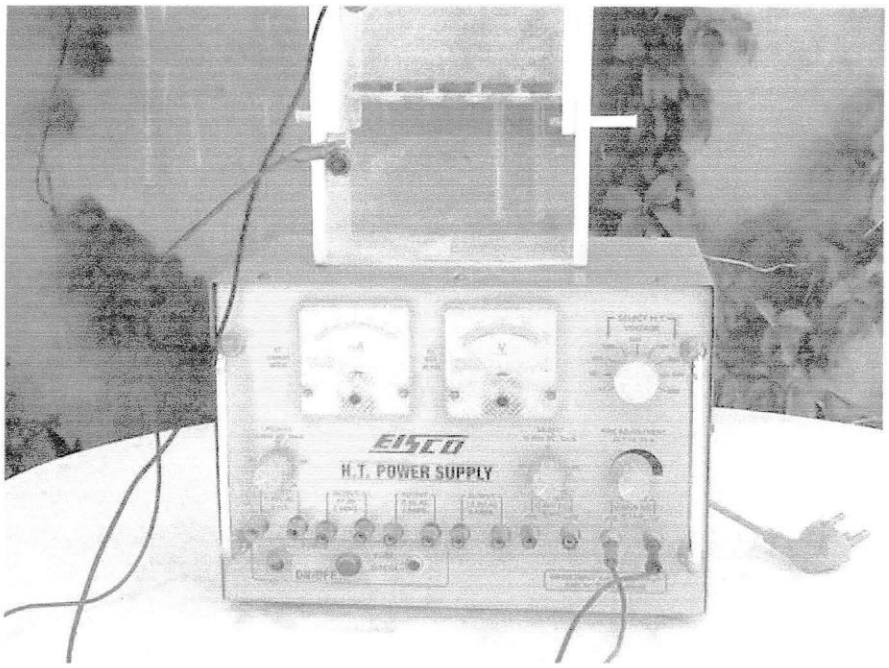


Figure (2.3): Disk gel electrophoresis system

Gels containing 3% (stacking gel), 8% or 10% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% of N,N′-bis-methylene acrylamide. The final concentration in the separation gel were as follows: 0.373 M Tris-HCL (pH 8.8) and 0.1% SDS. The gel was polymerized chemically by the addition of 0.025% by volume of tetramethylethylenediamine (TEMED) and ammonium persulphate.

Ten cm gels were prepared in glass tubes of a total length of 15 cm and with an inside diameter of 6 mm. The stacking gels of 3% acrylamide and a length of 1 cm contained 0.125 M Tris-HCL (pH 6.8) and 0.1% SDS were polymerized chemically in the same way as for the separating gel. The electrode buffer (pH 8.3) contained 0.025 M Tris and 0.192 M glycine and 0.1% SDS. The samples (0.2-0.3) ml contained the final concentrations ("final sample buffer"): 0.0625 M Tris-HCL (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue as the dye.

The proteins (PRL samples) were completely dissociated by immersing the samples for 1.5 minutes in boiling water.

Electrophoresis was carried out with a current of 3 mA per gel until the bromophenol blue marker reached the bottom of the gel (about 7 hours). The proteins were fixed in the gel with 50% trichloroacetic acid (TCA) overnight, stained for 1 hour at 37°C with a 0.1% Coomassie brilliant blue S-250 solution made up freshly in 50% TCA. The gels were diffusion-destained by repeated washing in 7% acetic acid (Laemmli 1970). A protein standard kit (Promega; Low and high-range protein molecular weight markers ranging from 14 up to 150 kDa molecular weight) were used and applied for gel electrophoresis at the same time of sample processing.

2.9. Specimen Collection and Preparation for Prolactin Receptor Analysis:

Routinely processed, formalin fixed paraffin embedded tissues is suitable for use with the monoclonal antibody to prolactin receptor (quartett Cat. # 161803701) when used with BIOCARE detection kit and DAB500 Chromogen System (Cat. # DB801R). The recommended tissue fixative is 10% neutral buffered formalin (Sheehan and Hrapchak 1980).

Each section should be cut at approximately 5µm thickness and placed on positively charged glass slid.

Staining of formalin/paraffin tissues requires digestion with pepsin 1mg/ml Tris-HCL. Cat. # 401603799 for 15 min at room temperature.

Chapter Three

Results

3. Results:

In this study the data were based on the analyses of 2 study groups:

First group (G1): consist of 53 out of 62 leiomyoma (LM) patients (85.48%), age range (31-49) years. All patients included in this group were with normal pituitary image.

None of those patients was on any drugs known to increase serum prolactin level in the last 6 months.

None of them was known to complain of diabetes mellitus, pituitary, and thyroid, renal or psychiatric disease.

Eighteen patients (33.96%) were under hysterectomy and 35 patients (66.04%) were under myomectomy.

Eighty-six leiomyoma samples with different uterine sites were harvested from 53 patient women.

Second group (G2): Forty pregnant women were taken as a normal control group underwent cesarean for maternal or fetal cause comparable to their age group, age range between 19-45 years and mean age was 32 years. None of them had leiomyoma (which was confirmed by ultrasonagraphy).

3.1. Risk factors associated with developing uterine leiomyoma:

Table (3.1) shows risk factors associated with developing uterine leiomyoma. The mean age for LM patients and age of menarche were found (39.9±5.5) (13.1±0.6) years respectively.

All LM patients were more frequently overweight/obese; their mean BMI was (28.4±4.7) Kg/m^2.

None of patients were smokers, and they were all not on an oral contraceptive (OC).

Nine out of 53 LM patients (16.98%) were single, while 44 (83.02%) were married. Thirty-one out of 44 married patients in this study had 1-4 times parity which represents the highest percentage among them (70.5%). Only 8 patients had 5 or more times parity (18.2%) while the lowest percentage was among those 5 LM patients with nulliparous (11.3%).

Table (3.1): Distribution of the study sample by mean and percentage of different variables.

	Mean±Std.D.	***Percentage (%)***
Age (years)	39.9±5.5	
Age at menarche (years)	13.1±0.6	
BMI (Kg/m^2)	28.4±4.7	
Smoking	------	-----
Oral contraceptive users	------	-----
Marital status		
Single	------	9 (16.98)
Married	------	44 (83.02)
Total patients		***53 (100.0)***
Parity		
Nulliparous	------	5 (11.3)
1-4	-----	31(70.5)
≥5	-----	8 (18.2)
Total Percentage		***44 (100.00)***

Std.D. =Standard Deviation

3.2. Prolactin in patient and control groups:

Table (3.2): Mean standard deviation of prolactin in serum, leiomyoma and myometrium of patient group comparing with mean standard deviation of prolactin in control myometrium.

		N	*Mean±std.D.*
Patient		53	
	Leiomyoma PRL (ng/ml)	86	18.2±14.0
	Myometrium PRL (ng/ml)	53	8.0±3.3
	Serum PRL (ng/ml)	53	143.1±106.9
*Control**	*myometrium PRL (ng/ml)*	40	6.7±2.6

Std.D.=Standard Deviation, (*) No serum sample was taken from this group because already the prolactin level in a pregnant woman is higher 10 folds than the normal.

Note: Mean serum prolactin for healthy menstrual women was found 18.6±2.3 ng/ml.

In table (3.2), LM patients shows a high mean value of PRL level in their serum which is equal to (143.1±106.9) ng/ml compared with normal serum PRL level range in healthy menstrual women (5-35 ng/ml).

The mean PRL level of LM was greater than the PRL of myometrium in same patient group (18.2±14.0) (8.0±3.3) ng/ml respectively, while mean of PRL in the myometrium of control group was found (6.7±2.6) ng/ml.

3.2.1. The relation between PRL levels in both patients and control tissues:

By using the *t-test*, table (3.3) shows the amount of significance value found between PRL among tissues of patient and control groups.

Table (3.3): Sample *t-test* between prolactin patient and control groups.

	P value
Uterine LM PRL + patient myometrium PRL (ng/ml)	< 0.0001
Uterine LM PRL + Control myometrium PRL (ng/ml)	< 0.0001
Patient myometrium PRL + Control myometrium PRL (ng/ml)	(NS)

(NS)= Not significant.

A highly significant value was found (P<0.0001) between the LM PRL and the myometrium PRL in same patient as shown in table (3.3). The positive correlation between LM PRL and myometrium PRL is shown in figure (3.1).

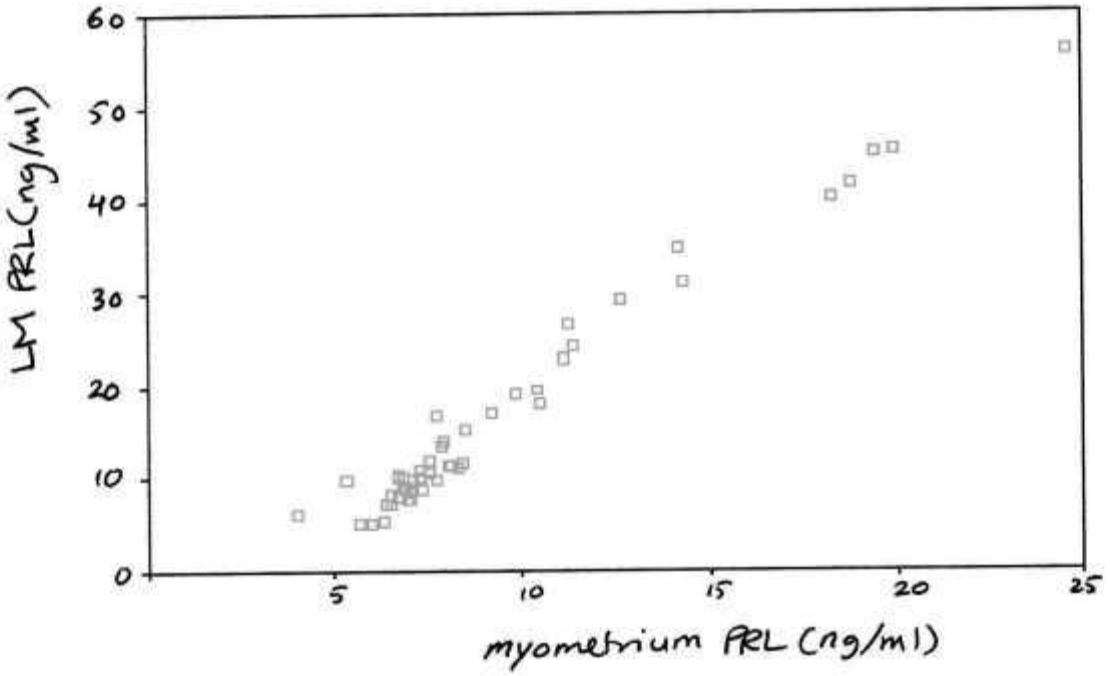


Figure (3.1): Scatter plot for correlation between LM PRL and myometrium PRL of same patient.

The relation between LM PRL and control myometrium PRL was highly significant (P<0.0001) (Table 3.3).

No significance was found between the PRL of patient myometrium and the control myometrium (Table 3.3).

3.2.2. The relation between leiomyoma prolactin, size and serum prolactin in patient group:

Table (3.4): Sample *t-test* between leiomyoma prolactin, size and patient serum prolactin.

	P value
Serum PRL(ng/ml) +LM PRL (ng/ml)	< 0.0001
Serum PRL (ng/ml) + LM size (cm^3)	< 0.0001
Serum PRL/LM PRL +LM size (cm^3)	< 0.0001
LM PRL (ng/ml)+LM size (cm^3)	0.015

A highly significant value was found between patients serum PRL and their LM PRL (P<0.0001) as shown in table (3.4). This significance had a positive correlation (Figure 3.2) when it applied in a scattered plot.

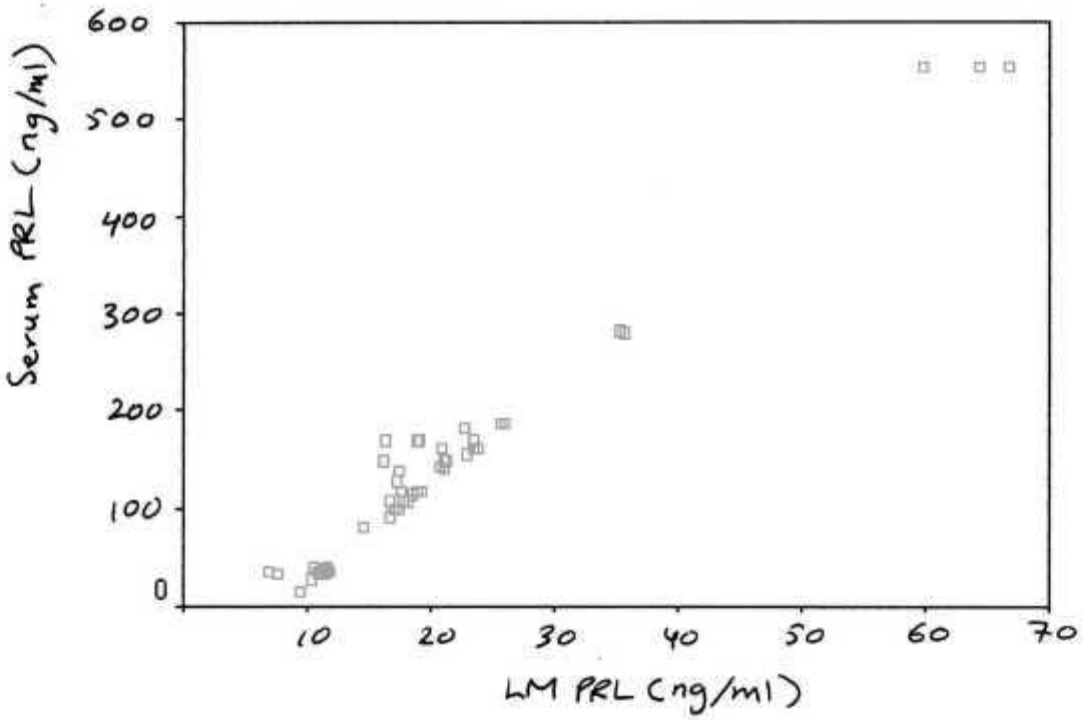


Figure (3.2): Scatter plot with positive correlation between patients serum PRL and their LM PRL.

Patient serum PRL also was found to have highly significant value ($P<0.0001$) with the size of LM (Table 3.4). And figure (3.3) shows the positive correlation between them in a scatter plot.

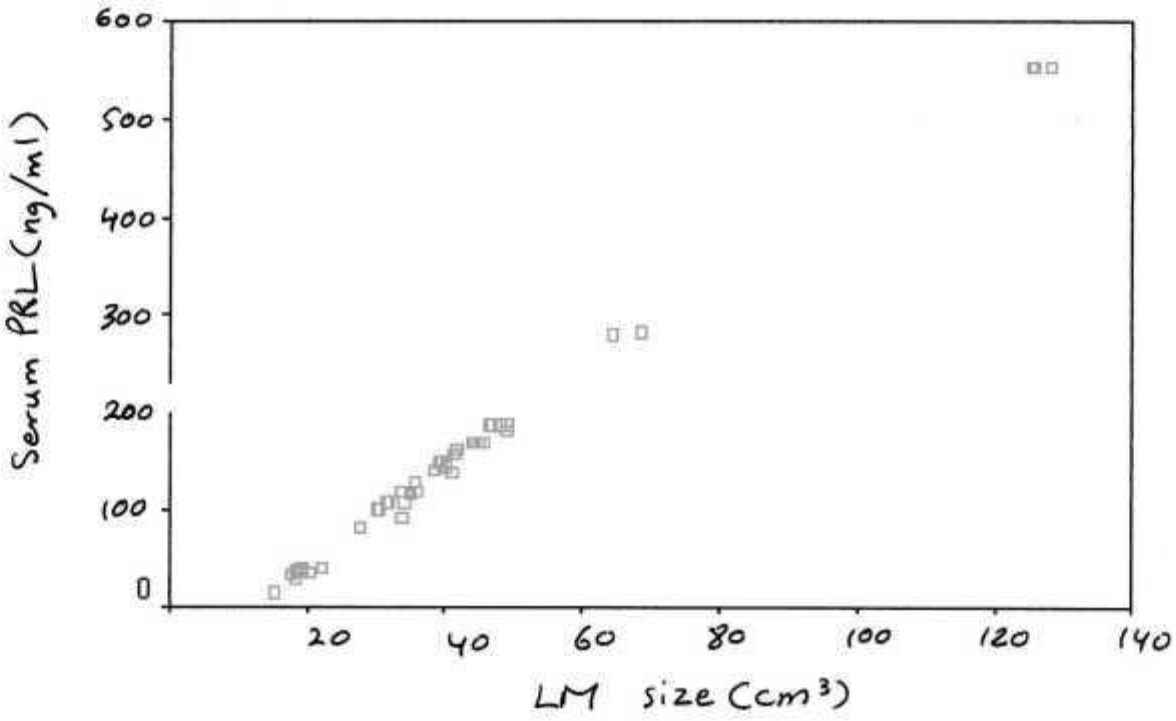


Figure (3.3): Scatter plot with positive correlation between serum PRL and the LM size.

Serum PRL to LM PRL ratio of same patient gives a highly significant value ($P<0.0001$) with the LM size (Table 3.4).

A significant value ($P<0.05$) was found between LM(s) PRL and their size. This significance had also a positive correlation as shown in figure (3.4).

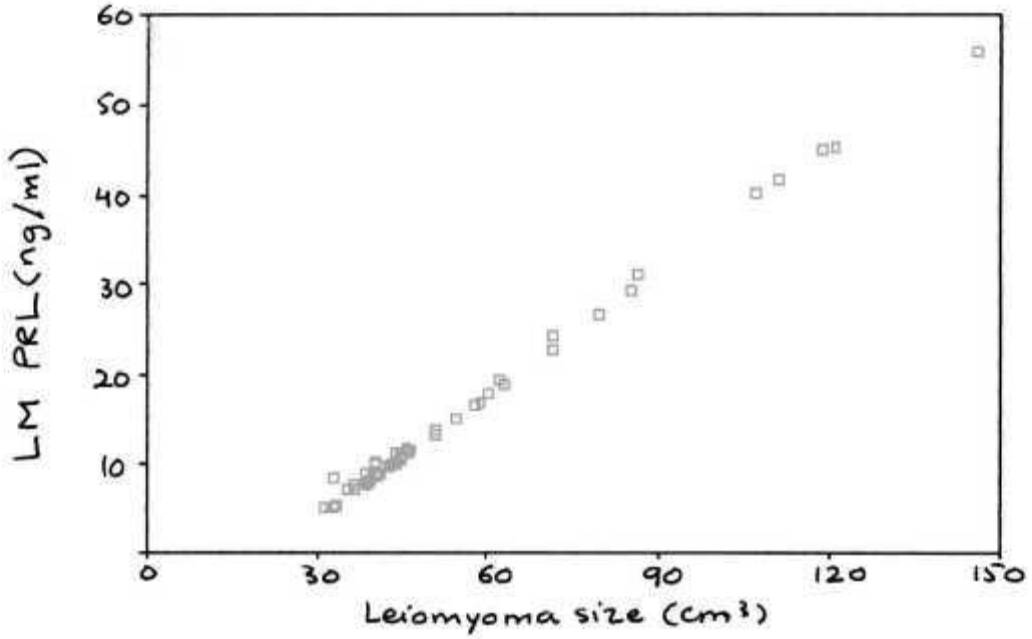


Figure (3.4): Scatter plot with positive correlation between LM PRL and LM size.

3.3. Prolactin profiles and their molecular weights in serum and tissues:

3.3.1. Prolactin profiles:

Table (3.5) shows the percentage of each PRL isoform found in serum, LM and myometrium of same patient group comparing with PRL isoform found in the myometrium of control group.

Table (3.5): Prolactin isoforms in both patient and control groups.

		Patient		*Control*
Prolactin isoforms	***Serum***	***Uterine leiomyoma***	***myometrium***	***myometrium***
Monomeric PRL	(30)56.6%	(63)73.3%	(45)84.9%	(32)80.0%
Big PRL	(22)41.5%	(23)26.7%	(8)15.1%	(8)20.0%
Big big PRL	(1)1.9%	----------	----------	---------
Total	**(53)100.0%**	**(86)100.0%**	**(53)100.0%**	**(40)100.0%**

The predominant PRL isoform found in this study was the monomeric PRL as shown in table (3.5). Monomeric PRL was found in both patient and control groups.

Serum of 30 patients out of 53 (56.6%) were with monomeric PRL, while (73.3%) and (84.9%) represents the percentage of monomeric PRL in their LM and myometrium respectively. Also 32 (80.0%) out of 40 control myomerium had monomeric PRL isoform.

Big PRL isoform represents the second PRL isoform found predominantly in both groups. 22 (41.5%) out of 53 LM patients had big PRL isoform in their serum. Tissues (LM and myometrium) of same patient group as well as myometrium of control group had also big PRL isoform with percentages (26.7%) (15.1%) and (20.0%) respectively.

Only one patient serum sample out of 53 had big big PRL isoform. And no big big PRL was found in tissues of patient and control groups.

3.3.2: Prolactin molecular weight:

To evaluate the molecular weight (MWt.) of each PRL isoform in both patient and control group, samples were underwent disk gel electrophoresis technique and then all separated result images were introduced in the PhotoCaptMWt. Program to calculate the PRL MWt.

Figure (3.5) shows the separated bands by disk gel electrophoresis technique.

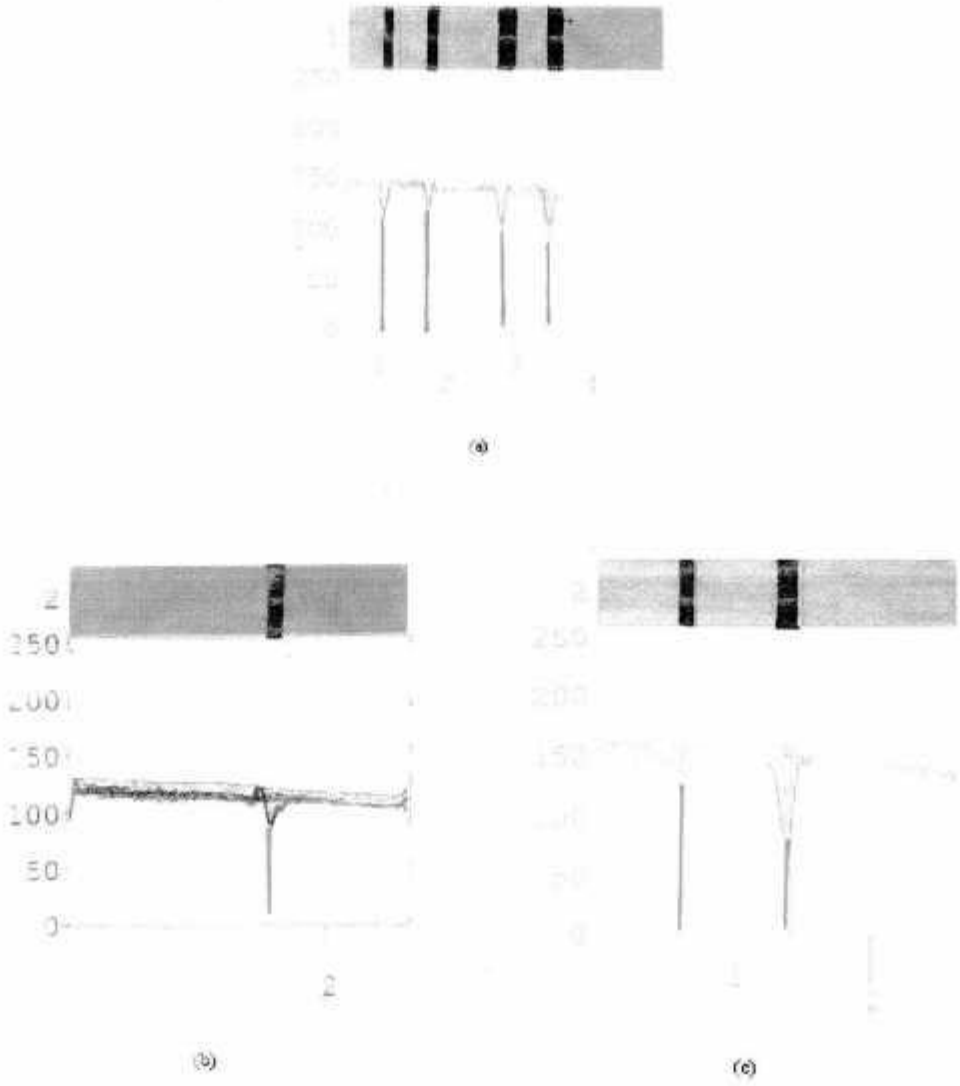


Figure (3.6): Disk electrophoresis images underwent PhotoCaptMWt. program for calculating the observed bands molecular weight.
(a): 1,2,3, and 4 represent protein standard bands (150, 80, 20, and 14) kDa respectively.
(b): image of single band of monomeric PRL isoform found in normal control myometrium sample.
(c): image of double bands (1 and 2) represented monomeric and big PRL bands respectively in normal control myometrium sample.

All calculated MWt. results were compared with the recovery PRL percentage [dividing the post-PEG PRL level by the pre-PEG PRL level] as shown in Table (3.6).

Table (3.6): Comparison between the recovery PRL percentage and the calculated molecular weight in both patient group and control group, were R%=recovery PRL percentage, MWt. = molecular weigh

		Monomeric		*Big*		*Bigbig*	
		R%	**MWt. (kDa)**	**R%**	**MWt. (kDa)**	**R%**	**MWt. (kDa)**
	Serum	77.6±8.2	24.9±9.7 16.5 ±4.5	53.6±7.7	71.6±15.7	19.05	201.5
Patient	***Leiomyoma***	80.5±8.3	16.5 ±5.0	59.5±4.4	70.5 ±11.2	----------	
	myometrium	89.2±5.2	16.7 ±6.9	62.9 ±15.4	78.5±14.9	----------	
Control	***myometrium***	87.0 ±2.7	16.7±5.7	54.9 ±8.5	74.4±13.2	----------	

As shown in table (3.6), serum, LM and myometrium of same patient group had monomeric PRL isoform. Two types of monomeric PRL isoforms were found in patients serum, [24.9±9.7 and 16.5 ±4.5] kDa. While the LM and myometrium PRL of same patient group had monomeric isoform with

Mwt. 16.5 ±5.0 and 16.7 ±6.9 kDa. Control myometrium was also found with 16.7±5.7 kDa molecular weight.

The big PRL isoform was found with 71.6±15.7, 70.5 ±11.2 and 78.5±14.9 kDa for serum, LM and myometrium respectively of same patient group.

Big PRL isoform was also found in control myometrium with Mwt. 74.4±13.2 kDa.

The only big big PRL isoform found in this study was in one patient's serum with Mwt. 201.5 kDa.

All these calculated Mwt(s) in serum and tissues of both study groups were matched with their expected isoforms from the recovery PRL percentage.

3.4. Correlation between different sites of leiomyoma with their sizes:

In this study, 86 LMs were collected from different uterine sites of 53 patients as shown in table (3.7).

Table (3.7): Sizes, sites of leiomyoma(s) in patient group.

Leiomyoma site	Patient Number	Leiomyoma Number	Leiomyoma Size range (cm^3)
Intramural	24	41	0.67-136.5
Subserosal	10	24	0.09-12.02
Submucousal	7	9	3.98-6.0
Broadligament	6	6	1-88.03
Cervical	6	6	56.0-56.05
Total	**53**	**86**	-----

Forty-one intramural LMs were collected from 24 patients, 24 subserosal LMs were taken from 10 patients, 9 submucousal LMs were taken from 7 patients, 6 broadligament LMs were collected from 6 patients while 6 cervical LMs were taken from 6 patients. Figure (3.7) shows the patient percent distributed according to their LM site.

Some patients may have LM(s) accumulate in one uterine site, while others may show LM(s) in many different sites.

The size of these LM(s) was ranging from 0.09 cm^3 to 136.5 cm^3 as showing in the same table.

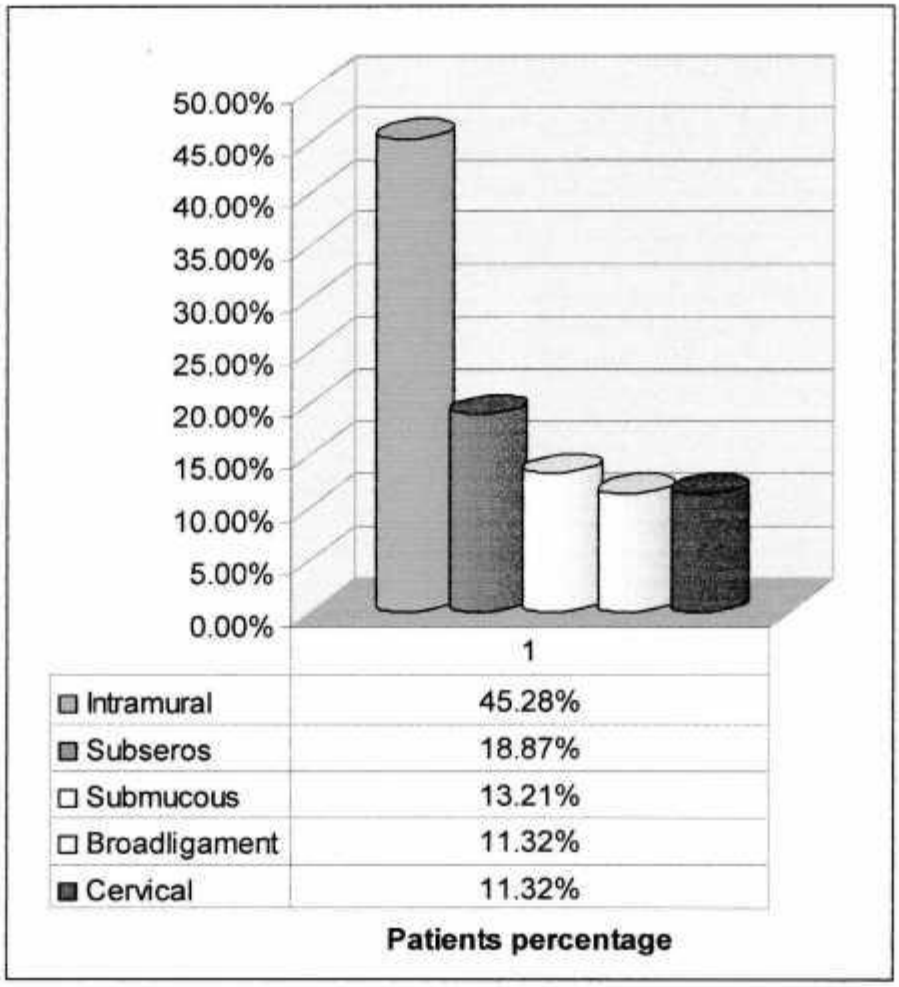


Figure (3.7): A histogram chart shows leiomyoma patient percent distributed according to their different leiomyoma sites.

Table (3.8) shows the correlation between different sites of LM with their sizes. It was found that there is a highly significant correlation between the subserosal, broadligament and cervical LM PRL with their sizes ($P< 0.01$), and a highly significant negative correlation between submucousal PRL and its size ($P<0.01$).

Also it was found that the submucousal PRL has a negative significant correlation with both cervical PRL and cervical size ($P<0.05$), while the submucousal size has a positive correlation with them ($P<0.05$). The broadligament PRL and its size has a significant correlation with cervical PRL and its size ($P<0.05$).

Table (3.8): Correlation between prolactin of different sites of leiomyoma with their sizes.

	Intramural PRL (ng/ml)	Intramural size (cm^3)	Subserosal PRL (ng/ml)	Subserosal Size(cm^3)	Submucousal PRL (ng/ml)	Submucousal Size(cm^3)	Broadligament PRL (ng/ml)	Broadligament Size(cm^3)	Cervical PRL (ng/ml)	Cervical size (cm^3)
Intramural PRL(ng/ml)										
Intramural size (cm^3)	-.134 .522									
Subserosal PRL (ng/ml)	-.117 .689	-.106 .719								
Subserosal size(cm^3)	.011 .969	-.151 .606	.807** .000							
Submucousal PRL (ng/ml)	.172 .658	-.152 .696	-.395 .293	.091 .816						
Submucousal size(cm^3)	-.112 .774	.072 .853	.461 .212	-.151 .698	-.976** .000					
Broadligament PRL (ng/ml)	.556 .252	.106 .842	.495 .318	-.623 .186	-.711 .113	.696 .125				
Broadligament size(cm^3)	.555 .253	.110 .836	.497 .316	-.623 .186	-.709 .114	.694 .126	1.000** .000			
Cervical PRL (ng/ml)	.571 .237	-.458 .361	.122 .818	-.540 .269	-.881* .021	.818* .046	.830* .041	.828* .042		
Cervical size (cm^3)	.571 .237	-.458 .361	.122 .818	-.540 .269	-.881* 021	.818* .046	.830* .041	.828* .042	1.000** .000	

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

3.5. Immunohistochemical study of prolactin receptor:

The haematoxylin (H) and eosin (E) stains were used to confirm the presence of uterine leiomyomas in tissue sections. Figure (3.8) shows the stained uterine leiomyomas sections of patients with their myometrium.

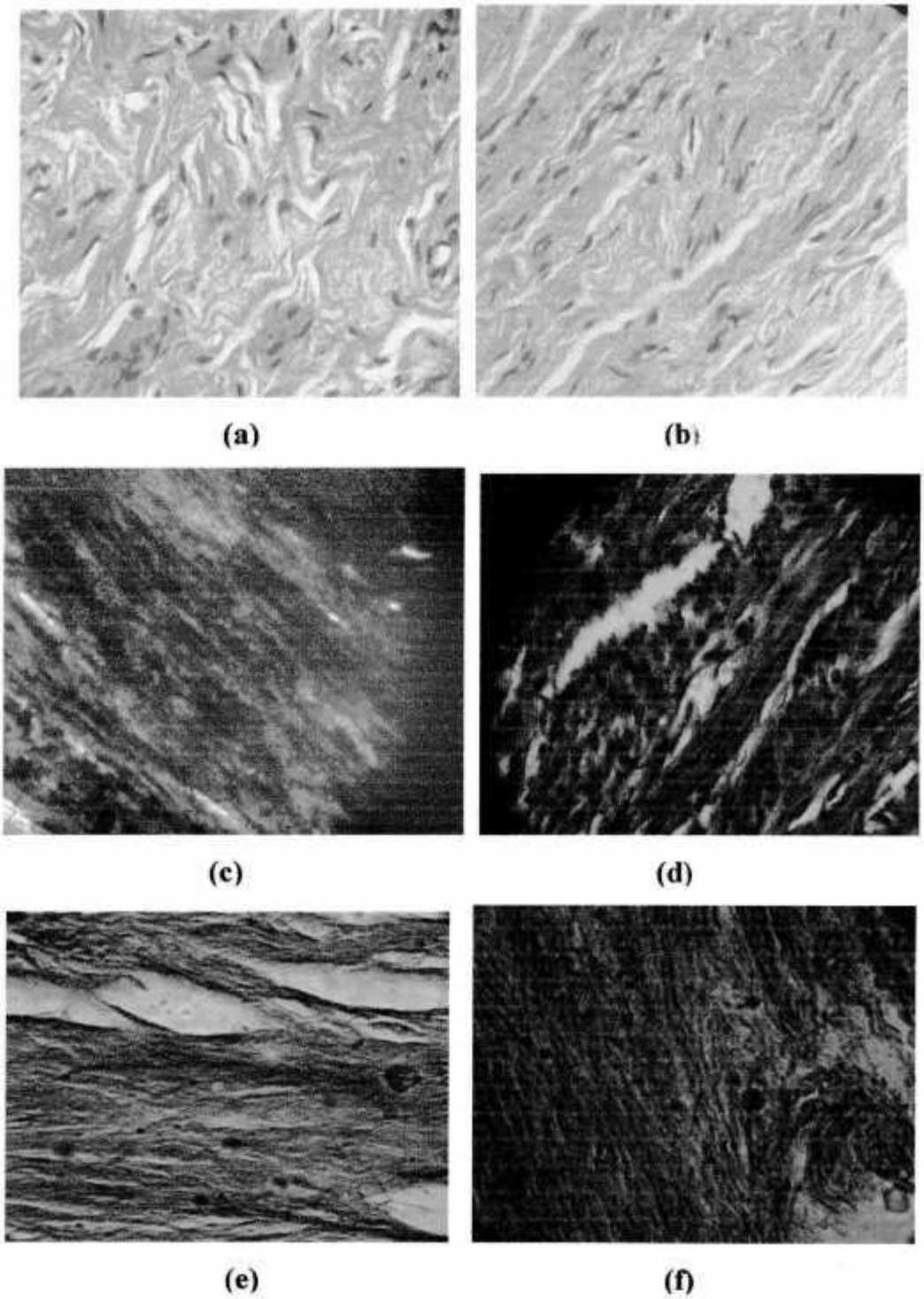
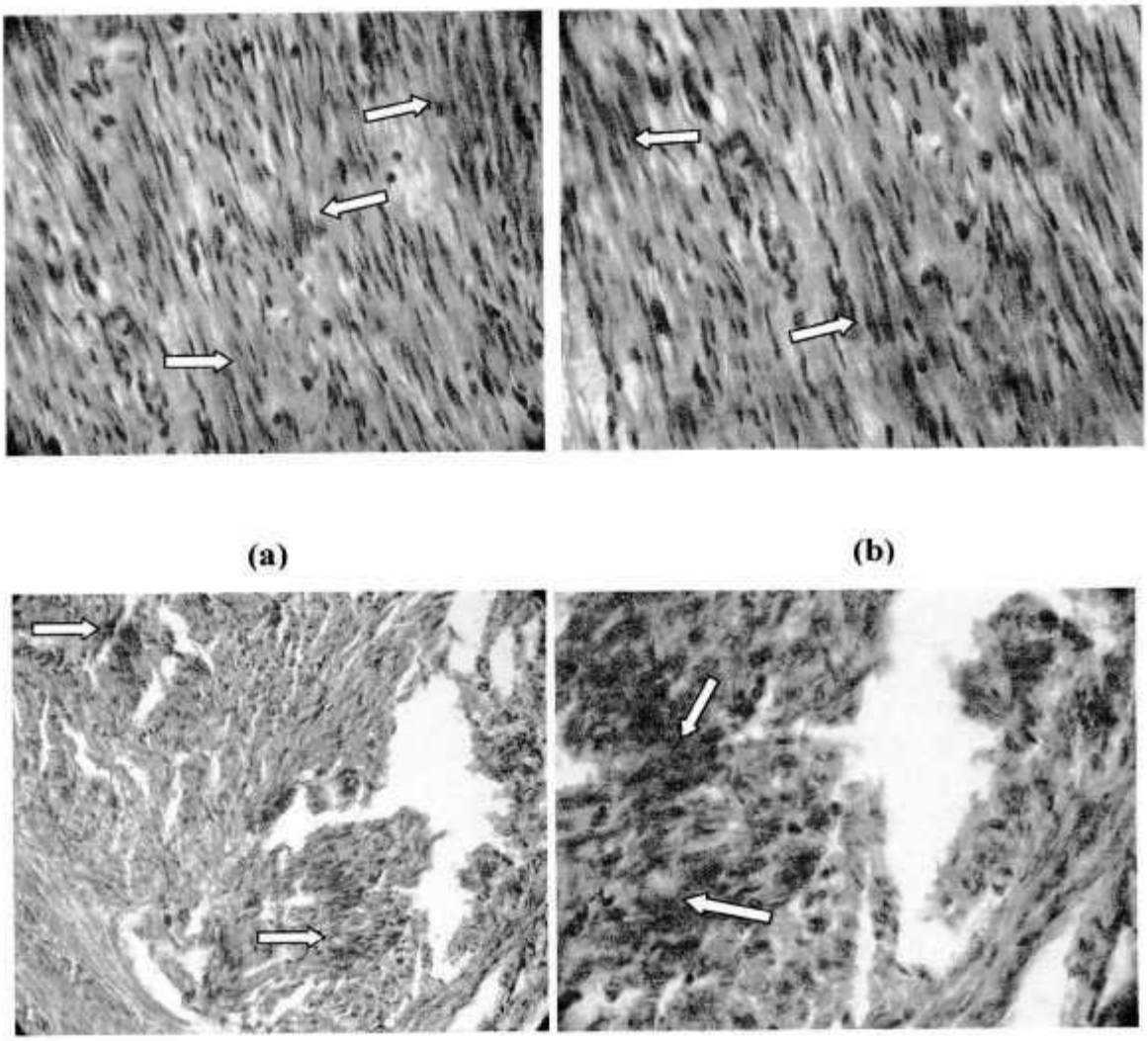


Figure (3.8): Haematoxylin and eosin stained sections for: (a, b, c, and d): uterine leiomyoma sections. (e): patient's myometrium. (f): control myometrium.

PRLR positivity was seen in all cases examined including patient and control tissues, in the form of dark brown staining (Figure 3.9) were formed as a result of reacting the monoclonal antibody of prolactin receptor with the extracellular portion of the receptor. Staining was heterogenous and varied in intensity from one case to another and sometimes from one area to another in the same section.



(a) (b)

(c) (d)

Figure (3.9): Brown spots indicated the location of prolactin receptors at tissues membrane using monoclonal antibody:
(a and b): uterine leiomyomas sections.
(c): patient myometrium section.
(d): control myometrium section.

4. Discussion :

4.1. Risk factors associated with developing uterine leiomyoma:

In this study as shown in table (3.1), the mean age of patient group was 39.9±5.5 years which refers that LM patients of this study were during their reproductive age (pre-menopausal). This result is agrees with Cramer and Patel, 1990, when they mentioned that uterine LM are the most common gynecologic neoplasm, occurring with a remarkable frequency in more than 70% of reproductive age women. Also Cheryl *et al.* 2001 reported that risk increases with age during the premenopausal years, but tumors typically regress and/or become asymptomatic with menopause. Gordon *et al.* 2003; refers to that estrogen has been traditionally proposed as the primary promoter of uterine leiomyoma growth. And this supposition was based in part upon the clinical observations that fibroids occur only after menarche and develop during the reproductive years.

Mean age at menarche was 13.1±0.6 years. This finding agrees with Cheryl 2002 who reported that at this early age there would be increase in overall exposure to circulating ovarian hormones and so increasing of risk associated with LM growth.

Leiomyoma patients in this study had mean BMI 28.4±4.7 kg/m^2. This mean value is approximately near the obese value ≥29 kg/m^2 [normal BMI=19-25 kg/m^2], were there is an increasing level of circulating estrogen through aromatization of fat stores. Cheryl 2002 also mentioned that obesity has been linked to an increased risk for LM. Also Ross *et al.* 1986 have mentioned that risk was strongly related to weight, women who weighed less than 55 kg had a particularly low risk, and overall the risk rose roughly 21% for each 10 kg.

None of this study patient was smoker, or being an OCs user. So, this will agree with increasing the risk for developing uterine LM, as Cheryl 2002 mentioned that smoking, use of OCs were identified as protection factors.

Ross *et al.* 1986 reported that those who smoke 20 cigarettes a day had a risk roughly two thirds that of non-smokers. Risk also decreases consistently with increasing duration of OC use; the risk of fibroids was found to be reduced by some 31% in women who had used OC for 10 years.

In this study, those 44 married patients have parity ranging from nulliparous to ≥5 parity. From table (3.1), the highest percentage (70.5%) was found among those patients with parity (1-4), while the lowest percentage (11.3%) was found in patients with nulliparous.

Ross *et al.* 1986 referred to pregnancy as one of risk factors associated with a decreased risk of developing LM, and in case of pregnancy, the risk of developing LM in parous women is approximately half of nulliparous women and it decreases the risk of fibroid with increased number of pregnancies. But Cheryl *et al.* 2001 said that studies that report changes in existing LM during pregnancy are not consistent. Some tumors grow, others shrink, but many show little change. Thus the mechanism(s) by which increasing parity might reduce the risk of fibroids are not understood, dissection of factors responsible is of importance, as the biological basis of this protective effect could yield valuable information with potential implications for therapy. The highest percentage found among those with 1-4 parity instead of nulliparous women is agrees with Cheryl *et al.* 2001 findings.

Lauren *et al.* 2004 also reported that overweight or obesity appeared to attenuate the inverse association between parity and uterine LM. This agreed with the finding of this study in which LM patients were

more likely overweight or obese and those with nulliparous or ≥5 parity had percentage lowest than those with 1-4 parity.

4.2. Prolactin in patient and control groups:

4.2.1. The relation between prolactin levels in patient and control tissue:

Mitchell *et al.* 1989 have reported that LM PRL secretion is significantly greater than myometrial PRL secretion for the same patient, and they found that LM PRL secretion increased with time whereas myometrial PRL secretion did not. This finding agrees with the mean results of this study as shown in table (3.2) in which the amount of PRL in LM was greater than myometrium PRL of the same patient and greater than the control myometrium PRL, while no significant differences was found between patient and control myometrium PRL. Also these results were confirmed by using the paired sample t-test as shown in table (3.3) and reached that there is a highly significant differences between LM PRL and myometrium PRL of the same patient as well as with the control myometrium ($P<0.0001$). No significance difference was found between patient and control myometrium PRL.

Daly *et al.* 1984 said that leiomyoma has the ability to synthesize prolactin which increases the evidence that cells of mesenchymal origin that arise near the paramesonephric ducts have a latent ability to express the genome for prolactin synthesis, and the appearance of prolactin synthesis in leiomyoma *in-vivo* suggests that this potential genome expression is activated either in smooth muscle cells or stromal cells during the transformation of normal cells to leiomyoma cells.

4.2.2. The relation between leiomyoma prolactin, size and serum prolactin in patient group:

As mentioned in table (3.4), there is a high relative association between the patients serum PRL and their LM PRL as well as between patients serum PRL with the LM size. Patient's LM sizes in this study were ranging between 0.15 cm^3 up to 100 cm^3. From these findings, serum of LM patient can be used as a marker for detecting the presence of fibroid and monitor its development depending on the level of serum PRL especially when it gives a high level in women with no pituitary adenoma.

In 1992, Chien *et al.*, have studied the ectopic production of PRL in uterine cervical carcinoma, they found that during the early stages of cancer, the degree of PRL elevation in serum and incidence of abnormal PRL levels are much greater than those found for carcinoembryonic antigen, which is generally considered to be a circulation cervical tumor marker. They considered that ectopic PRL production as a potential marker for detecting early occult tumor or for gauging the effectiveness of therapy for human cervical carcinoma.

Bhatavdekar *et al.* 2001 also studied the ectopic production of PRL by colorectal adenocarcinoma, and by assaying the circulating PRL and carcino embryonic antigen using immunoradiometric assay and radioimmunoassay kits, respectively in preoperative blood and tumor-draining venous blood samples of colorectal carcinoma patients. They had concluded that these multiple approaches confirmed that PRL is produced by colorectal carcinoma cells and by looking at its prognostic value and correlation with disease activity; it may provide a new insights into treatment for patients with colorectal carcinoma.

Also, Arnon *et al.* 2000 found that serum PRL levels decrease following hormonal or chemotherapy in patients with breast cancer. Therefore, they suggested that PRL may be used in the management and follow-up of patients with breast cancer by playing the role of a serum tumor marker.

4.3. Prolactin profiles and their molecular weights in serum and tissues:

4.3.1. Prolactin profiles:

Monomeric PRL was found to be the most predominant PRL isoform exist in LM patient and the control group (Table 3.5). This agrees with Ben-Jonathan *et al.* 1996 who mentioned that explants of normal myometrium as well as proliferative leiomyomas (fibroids) secreted immunoreactive PRL (monomeric isoform) into culture medium.

Bigbig PRL isoform was not found in patient tissues or control myometrium. But it was found only in one patient serum. This isoform did not appear lonely, it was found with both monomeric and big PRL isoforms in the same patient serum (Table 3.5).

This bigbig case can be considered as the first one found among LM patients. No cases or reports were found during the study search that had bigbig PRL isoform in LM patients.

In the 1980s, macroprolactinaemia was first identified as a new type of hyperprolactinemia. It was found to occur in 8±2.5% of patients with hyperprolactinaemia. This entity was defined by the bigbig PRL isoform being the only or the predominant form and was claimed to be poorly symptomatic and idiopathic. Although the nature of these large forms is still under debate a tumoral origin has been suggested (Leslie *et al.* 2001; Valette-Kasic *et al.* 2002).

It has been known that, patients with bigbig PRL do not show any clinical symptoms of hyperprolactinemia (those who have abnormal serum PRL level >35 ng/ml), such as amenorrhea, galactorrhea, and infertility, despite high hyperprolactinemic levels and they do not need specific treatment (Cavaco *et al.,* 1999), and because the predominant PRL isoforms were monomeric and big PRL, those forms were known to be active forms.

Suliman *et al.,* 2003, have reported that hyperprolactinemia is characterized by the presence of excess monomeric prolactin in serum. This finding agreed with serum results among the patient group whom have abnormal serum PRL level >35 ng/ml in this study.

They also said that macroprolactinemia can be defined by the presence of excess serum macroprolactin together with non pathologic monomeric PRL concentrations. So their finding agreed with the study result from which bigbig PRL (macroprolactin) isoform found in LM patient was together with monomeric and big PRL isoforms.

In addition to that, the macro PRL isoform is only found when there is large amount of PRL aggregation and conjugated with IgG. There is no reason can be given till now by researchers as an explanation about this inactive form, only that appearance of this isoform is due to the renal delay clearance which is due to its large molecule (Suliman *et al.,* 2003).

Corbacho *et al.,* 2002 have reported that PRL does not circulate as a single molecular species but as a family of related proteins. Circulating PRL in humans appears to consist of 5 isoforms: the classical 23 kDa molecule, a glycosylated PRL of 25 kDa, a 16 kDa fragment of PRL, dimmers of 50-60 kDa (big PRL), and aggregates of >100 kDa (bigbig PRL).

They mentioned that the actions of different members of the PRL family on angiogenesis provide one of the clearest examples directly

relating PRL functional diversity to its structural heterogenecity. So, full length PRL was considered to be inactive on blood vessel growth until showed its potential as a proangiogenic factor. Conversely, the enzymatically cleaved 16 KDa N-terminal fragment of PRL has a well defined anti-angiogenic effect.

4.3.2: Prolactin molecular weight in patient and control group:

Gel filtration technique was not used because it is difficult, expensive not recommended to be used nowadays (Mounier *et al.* 2003). It is also relatively insensitive requiring high levels of PRL, and since big and bigbig PRL forms represent only (6.1-42%) of the total PRL immunoreactivity and this agreed with what Blacker *et al.*, 1994 reported.

The predominant PRL isoform found in this study was the monomeric with molecular weight varying between 16 up to 25 kDa for both serum and tissues. This help explanation that PRL secreted from the LM is from the same source of smooth cell myometrium. The abnormal level of serum PRL in patient group, which is predominantly consisting of the monomeric with mean MWt. of PRL 16.5±4.5 kDa, is due to the ectopic PRL production of the LM, although, we can see, the monomeric MWt. in serum PRL found 24.9 kDa which is greater than that found in the tissues. Also it has big PRL isoform with mean MWt. of 71.6±15.7 kDa.

The 16 kDa prolactin is a PRL fragment retains PRL-like effects; it is mitogenic in the pigeon Crop-sac and in the Nb2 lymphoma cell bioassays. It has mammary mitogenic activity in the rat in vivo and it is

both mitogenic and lactogenic in rat mammary cells in culture (Clapp *et al.*, 1988).

16 kDa PRL could reach the circulation from different sources, including the pituitary gland and extra pituitary tissues (Corbacho *et al.* 2002).

The predominant PRL isoforms found in the control group were both monomeric and big PRL with mean MWt. of (16.7±5.7, 74.4±13.2) kDa respectively. And because this group consist of only pregnant women haven't any uterine fibroid, this result agreed with Corbacho *et al.* 2002 when reported that the concentration of 16 kDa PRL was elevated in pregnant women close to the day of delivery.

4.4. Correlation between different sites of leiomyoma with their sizes:

In this study, as shown in table (3.7), LMs collected from patients were from 5 different uterine sites. The highest percentage was for those patients with intramural LM, then those with subserosal and submucousal LM while both broadligament and cervical were found with the lowest percentage. The results then can be arranged in a descending manner as the following:

(High %) *intramural* LM > *subserosal* LM > *submucousal* LM > *broadligament* and *Cervical* LM ***(Low %)***. This agreed with (Sayyad 2004) when he reported that tumors in subserosal and intramural locations comprised the majority (95%) of all LMs; submucous LMs make up the remaining 5% although this classification scheme is widely used by clinicians, it suffers from the limitation that few LMs are actually a single "pure" type. Most LMs span more than one anatomic location

and, therefore, are hybrids (***e.g.***, a predominantly intramural LM with a submucous component).

From table (3.8), correlation between PRL of these different uterine LM sites and their sizes found with highly significant sometimes with positive correlation and other with negative one. And also it was found that there is a significant correlation between the PRL of LM sites with the size of another LM site with positive or negative correlation.

This confirms the idea that LM PRL functions as either autocrine or paracrine and so it affects the size of LM. Mitchell *et al.* 1989 explain their hypothesis that uterine PRL may be involved in the pathogenesis of fibroids. And similar to the other growth factors, uterine PRL may have important autocrine or paracrine regulatory functions. In 1999, Nowak *et al.* said in their report that PRL appeared to be an autocrine or paracine growth factor for both LM and myometrial cells. However, there are some differences between tissues in their sensitivity to these growth factors. They reach this conclusion after they study the effect of both (endogenous& exogenous) PRL as a mitogenic growth factor for human LM and myometrial cells.

4.5. Immunohistochemical study for prolactin receptor:

In this study, brown spots shows heterogeneity in there numbers and accumulation. Especially the difference was clearly obvious between accumulation and number of brown spots in the leiomyomas sections including their different sites and the myometrium sections from the same patient. The leiomyomas sections as shown in figure (3.12) had more distributed brown spots than myometrium of same patient. And the latent shows less accumulated brown spots with less distribution. While myometrium sections of control group (non-fibroid pregnant women) exhibit more accumulation and numbers of brown spots than those in

patient myometrium. The distributed spots may vary also from one area to another in the same tissue section especially in the myometrium sections.

Gill *et al.* (2001) studied the expression of PRLRs in normal, benign, and malignant breast tissue by an immunohistological method and they concluded that because prolactin plays an important role in the proliferation and differentiation of normal breast epithelium, they suggested that the higher expression of PRLR noted is in the cells of most benign and malignant breast lesions, compared with normal cells, could be an important factor in the pathogenesis of these diseases, rather than a reflection of the high proliferate activity of the abnormal cells.

They studied also the normal breast PRLR and they had seen positivity in all cases examined, in the form of dark brown staining of the luminal borders of the epithelial cells lining the ducts and acini. Staining was heterogenous and varied in intensity from one case to another and sometimes from one area to another in the same section. Myoepithelial cells were negative, in addition to fibrous tissue and blood vessels.

They mentioned also that there is a significant relation between the estrogen receptor (ER) and PRLR in female with invasive breast carcinoma ($P<0.05$).

Richards *et al.* (1999) have examined the estrogen receptor content of fibromyomata, in relation to the estrogen receptor content of their host myometria and normal myometria. Estrogen receptor levels in fibromyomata are thought to be influenced by the endometrial cycle, increasing through the proliferative phase into the early secretory phase before falling just prior to the onset of menses. They concluded that the increase in estrogen receptors in fibromyomata is expected given that the underlying host myometrium is abnormal. The phasic differences in receptor content may help explain the higher mitotic indices in tumors

from secretory phase uteri. Furthermore the differential staining patterns of the nuclei may be related to differences in gene regulation as a result of the fibromyomata's heightened sensitivity to estrogen.

4.6. Finding Summary:

- Age, obesity and age at menarche were risk factors associated with developing uterine leiomyoma, while increasing parity did not decrease this risk as it is known.
- Serum prolactin levels are increased with increasing leiomyomas prolactin and their sizes in patients with uterine leiomyoma, with a significant correlation.
- There is a correlation between leiomyomas prolactin and their sizes.
- Leiomyomas prolactin including their five different sites has a correlation with each other affecting their sizes. This result can be used as a start point to study the paracrine/autocrine of leiomyoma prolactin action.
- In addition to the known monomeric prolactin molecular weight in human serum, 16 kDa prolactin was found both in patient's serum and tissues (leiomyoma and myometrium).
- Both monomeric and big prolactin isoforms are responsible for increasing prolactin levels in patients' serum.
- Big big prolactin was found for the first time in uterine leiomyomas patient's serum which was only found in patients with pituitary adenoma.
- Prolactin receptor was detected for the first time in uterine leiomyomas sections.

5. Conclusions:

- Excess serum prolactin in leiomyoma patients comes from the leiomyoma itself and this is confirmed by the positive association between serum prolactin and leiomyoma prolactin and the presence of the same isoforms of this hormone.
- Age, obesity, age at menarche and leiomyoma size are modulating factors.
- The isolation of bigbig prolactin from one patient's serum for the first time needs further study.

References

References:

Adami HO, Signorello LB, Trichopoulos D. Towards an understanding of breast cancer etiology. Semin Cancer Biol 1998; 8: 255-262.

Alam NA, Bevan S, Churchman M, Barclay E, Barker K, Jaeger EE, *et al.* Localization of a gene (*MCUL1*) for multiple cutaneous leiomyomata and uterine fibroids to chromosome 1q42.3-q43. Am J Hum Genet 2001; 68:1264-1269.

Andersen J, Grine E, Eng CL, *et al.* Expression of connexin-43 in human myometrium and leiomyoma. Am J Obstet Gynecol 1993; 169:1266-1276.

Andersen J, DyReyes VM, Barbieri RL, *et al.* Leiomyoma primary cultures have elevated transcriptional response to estrogen compared with autologous myometrial cultures. J Soc Gynecol Invest 1995; 2: 542-551.

Aron D , Howlett T. Pituitary incidentalomas. Endocrinol Metab Clin North Am 2000; 29:205–221.

Barbieri RL, McShane PM, Ryan KJ. Constituents of cigarette smoke inhibit human granulosa cell aromatase. Fertil Steril 1986; 46:232-236.

Banerjee R, Ginsburg E, Vonderhaar BK. Characterization of a monoclonal antibody against human prolactin receptors. Int J Cancer 1993; **55**:712–21.

Baxter RC, Zaltsman Z, Turtle JR. Rat growth hormone (GH) but not prolactin (PRL) induces both GH and PRL receptors in female rat liver. Endocrinology 1984; 114:1893-901.

Ben-Jonathan N, Liby, K, McFarland M, *et al.* Prolactin as an autocrine/paracrine growth factor in human cancer. Trends Endocrinol. Metab. 2002; 13, 245–250.

Ben-Jonathan N, Mershon JL, Allen DL, *et al.* Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev 1996; 17:639-69.

Berlanga JJ, Gualillo O, Buteau H, *et al.* Prolactin activates tyrosyl phosphorylation of insulin receptor substrate 1 and phosphatidylinositol-3-OH kinase. J Biol Chem 1997; 272:2050-2.

Bhatavdekar JM, Patel DD, Chihlikar PR, *et al.* Ectopic production of prolactin by colorectal adenocarcinoma. Dis Colon Rectum 2001; 44(1): 119-27.

Bhatavdekar JM, Patel DD, Ghosh N, *et al.* Interrelationship of prolactin and its receptor in carcinoma of colon and rectum: a preliminary report. J Surg Oncol 1994; 55.

Blacker C, Feinstein MC, Avoine D, *et al.* Hétérogénéité de la prolactin: consequences cliniques. Gynécologie 1994; 2:352-357.

Blum V, Fiedler K. Hormonal control of reproductive behavior in some cichlid fish. Gen. Comp. Endocrinol 1965.; 5:186-196.

Bole-Feysot C, Goffin V, Edery M, *et al.*. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 1998; 19:225-68.

Bonneterre J, Peyrat JP, Beuscart R, *et al.* Prognostic significance of prolactin receptors in human breast cancer. Cancer Res 1987;47:4724–8.

Boutin JM, Jolicoeur C, Okamura H, *et al.* Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 1988; 53:69–77.

Boutin JM, Edery M, Shirota M, *et al.* Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Mol Endocrinol 1989; 3:1455-61.

Brosens I, Deprest J, Dal Cin P, *et al.* Clinical significance of cytogenetic abnormalities in uterine myomas. Fertil Steril 1998; 69:232-235.

Burtis CA, Ashwood ER. Tietz Text Book of Clinical Chemistry. 3rd edition. 1999. p.p. 18-19. W.B. Saunders Company.

Chien TH, Mu-Hsien Y, Chi-Yu GL, *et al.* Ectopic production of prolactin in uterine cervical carcinoma. Gynecology Oncology 1992; 44(2): 166-171.

Chow L, Lye SJ. Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. Am J Obstet Gynecol 1994; 170:788-795.

Clackson T, Ultsch MH, Wells JA, *et al.* Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity. J. Mol. Biol. 1998; 277:1111–1128.

Clapp C, Sears PS, Russell DH, *et al.* Biological and immunological characterization of cleaved and 16K forms of rat prolactin. Endocrinology 1988; 122: 2892-2898.

Clarke DL, Linzer DI. Changes in prolactin receptor expression during pregnancy in the mouse ovary. Endocrinology 1993; 133:224-32.

Clevenger CV, Kline JB. Prolactin receptor signal transduction. Lupus 2001; 10:706-18.

Clevenger CV, Thickman K, Ngo W, *et al.* Role of Bag-1 in the survival and proliferation of the cytokine-dependent lymphocyte lines, Ba/F3 and Nb2. Mol Endocrinol 1997; 11:608–618.

Cohen LM, Greenberg DB, Murray GB. Neuropsychiatric presentation of men with pituitary tumors (the 'four A's'). Psychosomatics 1984; 25:925-8.

Colditz GA. Epidemiology of breast cancer. Findings from the nurses' health study. Cancer 1993; 71: 1480-1489.

Corbacho AM, Martínez G, Clapp C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. J Endocrinol 2002; 173: 219-238.

Cramer SF, Patel A. The frequency of uterine leiomyomas. Am J Clin Pathol 1990; 94:435-438.

Cramer DW. Epidemiology of myomas. Semin Reprod Endocrinol 1992; 10:320-324.

Cramer SF, Horiszny JA, Leppert P. Epidemiology of uterine leiomyomas. With an etiologic hypothesis. J Reprod Med 1995; 40:595-600.

Dalrymple A. and Jabbour HN. Localization and Signaling of the Prolactin Receptor in the Uterus of the Common Marmoset Monkey.The Journal of Clinical Endocrinology & Metabolism 2000;85(4):1711-1718.

Daly DC, Walters CA, Prior JC, *et al.* Prolactin production from proliferative phase leiomyoma. Am J Obstet Gynecol 1984; 148:1059-1063.

Daniel M, Martin AD, Faiman C. Sex hormones and adipose tissue distribution in premenopausal cigarette smokers. Int J Obes Relat Metab Disord 1992; 16:245-254.

Das R, Vonderhaar BK. Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. Oncogene 1996; 13:1139–1145.

Davis JA, Linzer DI. Expression of multiple forms of the prolactin receptor in mouse liver. Mol Endocrinol 1989; 3:674-80.

Dawson R, Elliot DC, Elliott WH, *et al.* Data for biochemical research. 2nd edition .Clarendon press. Oxford. pp. 494., 1978.

De Placido S, Gallo C, Perrone F, *et al.* Prolactin receptor does not correlate with oestrogen and progesterone receptors in primary breast cancer and lacks prognostic significance. Ten year results of the Naples adjuvant (GUN) study. Br J Cancer 1990 ; 62:643–6.

De Schepper J, Schiettecatte J, Velkeniers B, *et al.* Clinical and biological characterization of macroprolactinemia with and without prolactin–IgG complexes. European Journal of Endocrinology 2003; 149: 201–207.

Dixon D., He H., and. Haseman JK. Immunohistochemical Localization of Growth Factors and Their Receptors in Uterine Leiomyomas and Matched Myometrium. Environmental Health perspectives Supplements 2000; 108(S5):1-9.

Djiane J, Durand P. Prolactin-progesterone antagonism in self regulation of prolactin receptors in the mammary gland. Nature 1977; 266:641-3.

Dombrowicz D, Sente B, Closset J, *et al.* Dose-dependent effects of human prolactin on the immature hypophysectomized rat testis. Endocrinology 1992; 130:695-700.

El-Garf A, Salah S, Shaarawy M, *et al.* Prolactin hormone in juvenile systemic lupus erythematosus: a possible relationship to disease activity and CNS manifestations. J Rheumatol 1996 ; 23:374–377.

Elskins, PA, Christinger, HW, Sandowski, Y, *et al.* Ternary complex between placental lactogen and the extracellular domain of the prolactinreceptor. Nature Struct. Biol. 2000; 7: 808–815.

Franchesschi S. Role of reproductive factors on the risk of endometrial cancer. Int J Cancer 1998; 76: 784-786.

Freeman ME, Kanyicska B, Lerant A, *et al.* Prolactin: structure, function, and regulation of secretion. Physiol Rev 2000; 80:1523-631.

Freemark M, Avril I, Fleenor D, *et al.* Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. Endocrinology 2002; 143:1378-85

Freemark M, Fleenor D, Driscoll P, *et al.* Body weight and fat deposition in prolactin receptor-deficient mice. Endocrinology 2001; 142:532-7.

Friedman AJ, Harrison-Atlas D, Barbieri RL, *et al.* A randomized, placebo-controlled, double-blind study evaluating the efficacy of leuprolide acetate depot in the treatment of uterine leiomyomata. Fertil Steril 1989; 51:251-256.

Gambone JC, Reiter RC, Lench JB, *et al.* The impact of a quality assurance process on the frequency and confirmation rate of hysterectomy. Am J Obstet Gynecol 1990; 163:545-550.

Gill S, Peston D, Vonderhaar BK *et al.* Expression of prolactin receptors in normal, benign, and malignant breast tissue: an immunohistological study. J. Clin. Pathol 2001;54;956-960.

Glass AR. Endocrine aspects of obesity. Med Clin North Am 1989; 73:139-160.

Goffin V, Binart N, Touraine P, *et al.* Prolactin: the new biology of an old hormone. Annu Rev Physiol 2002; 64:47-67.

Goffin V, Shiverick KT, Kelly PA, *et al.* Sequence-function relationships within the expanding family of prolactin, growth hormone, placental lactogen and related proteins in mammals. Endocr Rev 1996; 17:385–410.

Gordon PF, Janet A, Darlene D. Etiology and pathogenesis of uterine leiomyomas: A Review. Environmental Health Perspectives 2003; 111:1037-1054.

Greenman Y, Tordjman K, Stern N. Increased body weight associated with prolactin secreting pituitary adenomas: weight loss with normalization of prolactin levels. Clin Endocrinol 1998 ; 48:547-53.

Haney AF. Clinical decision making regarding leiomyomata: what we need in the next millennium. Environ Health Perspect 2000; 108:835-839.

Hashimoto K, Azuma C, Kamiura S, *et al.* Clonal determination of uterine leiomyomas by analyzing differential inactivation of the X-chromosome-linked phosphoglycerokinase gene. Gynecol Obstet Invest 1995; 40:204-208.

Hattori N, Inagaki C. Anti-prolactin (PRL) autoantibodies cause asymptomatic hyperprolactinemia: bioassay and clearance studies of PRL-immunoglobulin G complex. J Clin Endocrinol Metab 1997 ; 82:3107–3110.

Hauache O, Rocha A, Maia Jr A, *et al.* Screening for macroprolactinaemia and pituitary imaging studies. Clin Endocrinol 2002; 57:327–331.

Hayden TJ, Bonney RC, Forsyth IA. Ontogeny and control of prolactin receptors in the mammary gland and liver of virgin, pregnant and lactating rats. J Endocrinol 1979; 80:259-69.

Hennighausen L, Robinson GW, Wagner K-U, *et al.* Prolactin signaling in mammary gland development. J Biol Chem 1997; 272;7567–9.

Hu ZZ, Meng J, Dufau ML. Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. J Biol Chem 2001; 276:41086-94.

Hu ZZ, Zhuang L, Meng J, *et al.* The human prolactin receptor gene structure and alternative promoter utilization: the generic promoter hPIII and a novel human promoter hP(N). J Clin Endocrinol Metab 1999; 84:1153-6.

Hu ZZ, Zhuang L, Meng J, *et al.* Complex 5' genomic structure of the human prolactin receptor: multiple alternative exons 1 and promoter utilization. Endocrinology 2002; 143:2139-42.

Jahn GA, Edery M, Belair L, *et al.* Prolactin receptor gene expression in rat mammary gland and liver during pregnancy and lactation. Endocrinology 1991; 128:2976-84.

Jarrett JCn, Ballejo G, Saleem TH, *et al.* The effect of prolactin and relaxin on insulin binding by adipocytes from pregnant women. Am J Obstet Gynecol 1984; 149:250-5.

Josimovich JB, Merisko K, Boccella L. Amniotic prolactin control over amniotic and fetal extracellular fluid water and electrolytes in the rhesus monkey. Endocrinology 1977; 100:564–570.

Keeler C, Dannies PS, Hodsdon ME. The tertiary structure and backbone dynamics of human prolactin. J Mol Biol 2003; 328:1105-1121.

Kelsey JL, Bernstein L. Epidemiology and prevention of breast cancer. Annu Rev Public Health 1996; 17: 47-67.

Kiuru M, Launonen V, Hietala M, *et al.* Familial cutaneous leiomyomatosis is a two-hit condition associated with renal cell cancer of characteristic histopathology. Am J Pathol 2001; 159:825-829.

Kjerulff KH , Langenberg P, Seidman JD, *et al.* Uterine leiomyomas. Racial differences in severity, symptoms and age at diagnosis. J Reprod Med 1996; 41:483-490.

Kline JB, Clevenger CV. Identification and characterization of the prolactin-binding protein in human serum and milk. J Biol Chem 2001; 276:24760-6.

Kline JB, Moore DJ, Clevenger CV. Activation and association of the Tec tyrosine kinase with the human prolactin receptor: mapping of a Tec/Vav1-receptor binding site. Mol Endocrinol 2001; 15:832-41.

Kline JB, Roehrs H, Clevenger CV. Functional characterization of the intermediate isoform of the human prolactin receptor. J Biol Chem 1999; 274:35461-8.

Kloehn S, Otte C, Korsanke M, *et al.* Expression and distribution of the prolactin receptor in normal rat liver and in experimental liver cirrhosis.Hoerm.Metab.Res2001;33(7):394-401.

Kulczycki LL, Robertson MT. The sweat chloride concentration and prolactin activity in cystic fibrosis. Scand J Gastroenterol 1988; 143:28–30.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(15): 680-85.

Lahat N, Miller A, Shtiller R, *et al.* Differential effects of prolactin upon activation and differentiation of human B lymphocytes. J. Neuroimmunol. 1993; 47:35-40.

Lambe M, Wuu J, Weiderpass E, *et al.* Childbearing at older age and endometrial cancer risk (Sweden). Cancer causes Control 1999; 10: 43-49.

Landgraf R, Landraf-Leurs MM, Weissmann A, *et al.* Prolactin: a diabetogenic hormone. Diabetologia 1977; 13:99-104.

Launonen V, Vierimaa O, Kiuru M, *et al.* Inherited susceptibility to uterine leiomyomas and renal cell cancer. Proc Natl Acad Sci USA 2001; 98:3387-3392.

Lauren AW, Julie RP, Bernard LH, *et al.* Reproductive factors, Hormonal contraception, and Risk of uterine leiomyomata in African-American Women: A prospective study. Am J Epidemiol 2004; 159:113-123.

Leslie H, Courtney C, Bell P, *et al.* Laboratory and clinical experience in 55 patients with macroprolactinemia identified by a simple polyethylene glycol precipitation method. J Clin Endocrinol Metab 2001; 86:2743-2746.

Levy B, Mukherjee T, Hirschhorn K. Molecular cytogenetic analysis of uterine leiomyoma and leiomyosarcoma by comparative genomic hybridization. Cancer Genet Cytogenet 2000 ;121:1-8.

Lindstedt G. Endogenous antibodies against prolactin – a 'new' cause of hyperprolactinemia. European Journal of Endocrinology 1994; 130: 429–432.

Ling C. Prolactin receptor exprecssion and prolactin-mediated effects in adipose tissue. Department of Physiology and Pharmacology Gteborg University, Sweden Gteborg.2002.

Liu X, Robinson GW, Gouilleux F, *et al.* Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc Natl Acad Sci USA 1995; 92: 8831-5.

Lumbiganon P, Rugpao S, Phandhu-fung S. Protective effect of depot-medroxyprogesterone acetate on surgically treated uterine leiomyomas: a multicentere case–control study. Br. J. Obstet. Gynaecol.1996; 103: 909–914.

Machida T, Taga M, Minaguchi H. Effect of Prolactin (PRL) on lipoprotein lipase (LPL) activity in the rat fetal liver. Asia Oceania J. Obstet. Gynaecol 1990.; 16: 261-265.

Mangrulkar RS, Ono M, Ishikawa M, *et al.* Isolation and characterization of heparin-binding growth factors in human leiomyomas and normal myometrium. Biol Reprod 1995; 53:636-646.

Marshall S, Bruni JF, Meites J. Prolactin receptors in mouse liver:species differences in response to estrogenic stimulation. Proc Soc Exp Biol Med 1978; 159:256-9.

Marshall LM, Spiegelman D, Barbieri RL, *et al.* Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. Obstet Gynecol 1997; 90:967-973.

Marshall LM, Spiegelman D, Goldman MB, *et al.* A prospective study of reproductive factors and oral contraceptive use in relation to the risk of uterine leiomyomata. Fertil Steril 1998; 70:432-439.

Mashal RD, Fejzo MLS, Friedman AJ, *et al.* Analysis of androgen receptor DNA reveals the independent clonal origins of uterine leiomyomata and the secondary nature of cytogenetic aberrations in the development of leiomyomata. Genes Chromosomes Cancer 1994; 1:1-6.

Matsuda M, Mori T, Sassa S, *et al.* Chronic effect of hyperprolactinemia on blood glucose and lipid levels in mice. Life Sci 1996; 58:1171-7.

McMurray RW, Allen SH, Pepmueller PH, *et al.* Elevated serum prolactin levels in children with juvenile rheumatoid arthritis and antinuclear antibody seropositivity. J Rheumatol 1995; 22:1577–1580.

McWilliams RB. Reproductive Endocrinology and Gynecology. http//:www.houstoninfertilityclinicfertilitytreatment. 2005.

Meloni AM, Surti U, Contento AM, *et al.* Uterine leiomyomas: cytogenetic and histologic profile. Obstet Gynecol 1992 ; 80:209-217.

Mitchell SR, Andrew JF, Linda JH. Decreased prolactin secretion by explant cultures of fibroids from women treated with a gonadotropin-Releasing Hormone Agonist. J Clin Endocrinol Metab 1989; 70(6): 1554-1558.

Moldrup A, Petersen ED, Nielsen JH. Effects of sex and pregnancy hormones on growth hormone and prolactin receptor gene expression in insulinproducing cells. Endocrinology 1993; 133:1165-72.

Molina PE. Endocrine physiology. Lange physiology series books/ McGraw-Hill. Page 67,2004.

Mounier C, Trouillas J, Claustrat B, *et al.* Macroprolactinemia associated with prolactin adenoma. Human Reproduction 2003; 18(4): 853-857.

Murphy AA, Kettel LM, Morales AJ, *et al.* Regression of uterine leiomyomata in response to the antiprogesterone RU 486. J Clin Endocrinol Metab 1993; 76:513-517.

Nag S, Sanyal S, Ghosh KK, *et al.* Prolactin suppression and spermatogenic developments in maturing rats. A quantitative study. Horm. Res. 1981; 15:72-77.

Nagy E, Berczi I. Hypophysectomized rats depend on residual prolactin for survival. Endocrinology 1991; 128: 2776–2784.

Nevalainen MT, Valve EM, Ingleton PM, *et al.* Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. Endocrinology 1996; 137:3078-88.

Neville MC, Daniel CW. The Mammary Gland: Development, Regulation and Function. Plenus, Press, New York,1987.

Nicoll CS, Mayer GL, Russell SM. Structural features of prolactins and growth hormones that can be related to their biological properties. Endocr Rev 1986; 7:169–203.

Nilbert M, Heim S. Uterine Leiomyoma Cytogenetics. Genes Chromosomes Cancer 1990; 2:3-13.

Nowak RA. Fibroids: pathophysiology and current medical treatment. Baillieres Best Pract Res Clin Obstet Gynaecol 1999; 13:223-238.

Nowak RA, Mora S, Diehl T, *et al.* Prolactin is an autocrine or paracrine growth factor for human myometrial and leiomyoma cells. Gynecol Obstet Invest 1999; 48:127-132.

Nowak RA, Rein MS, Heffner LJ, *et al.* Production of prolactin by smooth muscle cells cultured from human uterine fibroid tumors. J Clin Endocrinol Metab 1993; 76:1308–1313.

Olukoga A, Kane J. Macroprolactinaemia: validation and application of the polyethylene glycol precipitation test and clinical characterization of the condition. Clin Endocrinol 1999; 51:119–126.

Packenham JP, du Manoir S, Schrock E, *et al.* Analysis of genetic alterations in uterine leiomyomas and leiomyosarcomas by comparative genomic hybridization. Mol Carcinog 1997;19:273-279.

Pandis N, Heim S, Bardi G, *et al.* Parallel karyotypic evolution and tumor progression in uterine leiomyoma. Genes Chromosomes Cancer 1990; 2:311-317.

Parazzini F, La Vecchia C, Negri E, *et al.* Epidemiologic characteristics of women with uterine fibroids: a case-control study. Obstet Gynecol 1988; 72:853-857.

Parazzini F, Negri E, La Vecchia C, *et al.* Uterine myomas and smoking. Results from an Italian study. J Reprod Med 1996; 41:316-320.

Parslov M, Lidegaard O, klintrop S, *et al.* Risk factors among young women with endometrial cancer: a Danish case-control study. Am J Obstet Gynecol 2000 ;182: 23-29.

Partridge RK, Hahnel R. Prolactin receptors in human breast carcinoma. Cancer 1979;43:643–6.

Pesce and Strand. A new micromethod for determination of protein in cerebrospinal fluid and urine. Clin Chem 1973; 19(11); 1265-1267.

Pippard C, Baylis PH. Prolactin stimulates Na± K± ATP*ase* activity located in the outer renal medulla of the rat. J. Endocrinol.1986 ; 108: 95-99.

Patmastan P. Comparison of properties of wild-type human prolactin and a potent antagonist dissertation (PhD thesis). Graduate School of The Ohio State University,2003.

Posner BI, Kelly PA, Friesen HG. Prolactin receptors in rat liver: possible induction by prolactin. Science 1975; 188: 57-9.

Purvis, DH, Mabbutt BC. Solution dynamics and secondary structure of murine leukemia inhibitory factor: a four-helix cytokine with a rigid CD loop. Biochemistry 1997; 36: 10146–10154.

Pusztai L, Lewis CE, Lorenzen J, *et al.* Growth factors: regulation of normal and neoplastic growth. J Pathol 1993; 169:191-201.

Rambourg A, Clermont Y, Chretien M, *et al.* Formation of secretory granules in the golgi-apparatus of prolactin cells in the rat pituitary gland a stereoscopic study. Anat. Rec. 1992; 232: 169–179.

Rao R. Textbook of Biochemistry for Medical Students. 15th Revised Edition. New Delhi, Tanuku, A.P., India. pp 142, 1986.

Redfield C, Boyd J, Smith LJ, *et al.* Loop mobility in a 4-helixbundle protein—N-15 NMR relaxation measurements on human interleukin-4. Biochemistry 1992; 31: 10431–10437.

Rein MS, Friedman AJ, Heffner LJ. Decreased prolactin secretion by explant cultures of fibroids from women treated with a gonadotropin-releasing hormone agonist. J Clin Endocrinol Metab 1990; 70:1554-1558.

Rein MS, Powell WL, Walters FC, *et al.* Cytogenetic abnormalities in uterine myomas are associated with myoma size. Mol Hum Reprod 1998; 4:83-86.

Rein MS, Barbieri RL, Friedman AJ. Progesterone: a critical role in the pathogenesis of uterine myomas. Am J Obstet Gynecol 1995; 172:14-18.

Rein MS. Advances in uterine leiomyoma research: the progesterone hypothesis. Environ Health Perspect 2000; 108 (suppl 5): 791-793.

Reis FM, Reis AM, Coimbra CC. Effects of hyperprolactinaemia on glucose tolerance and insulin release in male and female rats. J Endocrinol 1997; 153:423-8.

Richards PA, Richards GDP, Shawki O.Estrogen receptor levels in fibromyomata. Middle East Fertility Society Journal 1999; 4(2): 129-33.

Richards PA, Tiltman AJ. Anatomical variation of the oestrogen receptor in the non-neoplastic myometrium of fibromyomatous uteri. Vichows Arch 1996; 428: 347-351.

Riddle O, Bates RW, Dykshorn SW. The preparation, identification and assay of prolactin - a hormone of the anterior pituitary. Am J Physiol 1933; 105:191–216.

Roberson MT, Boyajian MJ, Patterson K, *et al.* Modulation of the chloride concentration of human sweat by prolactin. Endocrinology 1986; 119: 2439-2444.

Romieu I, Walker AM, Jick S. Determinants of uterine fibroids. Post Market Surveill 1991; 5:119-133.

Ross RK, Pike MC, Vessey MP, *et al.* Risk factors for uterine fibroids: reduced risk associated with oral contraceptives. Br. Med. J. (Clin. Res. Ed.) 1986; 293: 359–362.

Rozakis-Adcock M, Kelly PA. Mutational analysis of the ligand-binding domain of the prolactin receptor. J Biol Chem 1991; 266:16472-7.

Rustin P, Bourgeron T, Parfait B, *et al.* Inborn errors of the Krebs cycle: a group of unusual mitochondrial diseases in human. Biochim Biophys Acta 1997; 1361:185-197.

Ryan EA, Enns L. Role of gestational hormones in the induction of insulin resistance. J Clin Endocrinol Metab 1988; 67:341-7.

Sage M. Control of prolactin release and its role in color change in the teleost Gillichthys mirabilis. J. Exp. Zool. 1970; 173: 121-127.

Saif Allah PH. Biochemical studies on prolactin and some tumor markers in breast tumors (PhD. Thesis). College of Science, University of Baghdad. pp.47, 2000.

Samadi AR, Lee NC, Flanders WD, *et al.* Risk factors for self-reported uterine fibroids: a case-control study. Am J Public Health 1996; 86: 858-862.

Sankoorikal BJ, Zhu YL, Hodsdon ME, *et al.* Aggregation of human wild-type and H27A-prolactin in cells and in solution:roles of Zn^{+2} ,Cu^{+2} , and pH . Endocrinology 2002; 143: 1302–1309.

Sarandakou A, Kassanos D, Phocas I, *et al.* Amniotic fluid hormone profiles during normal and abnormal pregnancy. Clin Exp Obstet Gynecol 1992; 19:180–188.

Sato F, Miyake H, Nishi M, *et al.* Early normal menstrual cycle pattern and the development of uterine leiomyomas. J Womens Health Gend Based Med 2000; 9:299-302.

Sauro MD, Zorn NE. Prolactin induces proliferation of vascular smooth muscle cells through a protein kinase C-dependent mechanism. J Cell Physiol 1991; 148:133-138.

Sayyad. What are fibroids? http://www.fibroids.net/html/frameset.htm., 2004.

Schernthaner G, Prager R, Punzengruber C, *et al.* Severe hyperprolactinaemia is associated with decreased insulin binding in vitro and insulin resistance in vivo. Diabetologia 1985 ; 28:138-42.

Schlechte JA. The macrprolactin problem. J Clin Endocrinol Metab 2002; 87(12):5408-5409.

Schneider J, Bradlow HL, Strain G, *et al.* Effects of obesity on estradiol metabolism: decreased formation of nonuterotropic metabolites. J Clin Endocrinol Metab 1983; 56:973-978.

Schneider W, Marcovitz S, Al-Shammari S, *et al.* Reactivity of macroprolactin in common automated immunoassays. Clin Biochem 2001; 34:469–473.

Schwartz SM, Marshall LM, Baird DD. Epidemiologic contributions to understanding the etiology of uterine leiomyomata. Environ Health Perspect 2000a; 108(suppl 5):821-827.

Schwartz SM, Voigt L, Tickman E, Yarbro P, *et al.* Familial aggregation of uterine leiomyomata. Am J. Epidemiol 2000b; 151: S10.

Sheehan DC, Hrapchak BB. Theory and practice of histotechnology, 2nd Edition. The C.V. Mosby Company, St. Louis, 1980.

Shewade DG, Ramaswamy S. Prolactin induced analgesia is dependent on ATP sensitive potassium channels. Clin Exp Pharmacol Physio 1995;l 22:635–636.

Smith CR , Norman MR. Prolactin and growth hormone: molecular heterogeneity and measurement in serum. Annals of Clinical Biochemistry 1990; 27: 542–550.

Smith TP, Suliman AM, Fahie-Wilson MN, *et al.* Gross variability in the detection of prolactin in sera containing big big prolactin (macroprolactin) by commercial immunoassays. Journal of Clinical Endocrinology and Metabolism 2002; 87: 5410–5415.

Sobrinho LG. The psychogenic effects of prolactin. Acta Endocrinol (Copenh) 1993; 129:38–40.

Sorenson RL, Brelje TC, Hegre OD, *et al.* Prolactin in vitro decreases the glucose stimulation threshold, enhances insulin secretion, and increases dye coupling among islet β-cells. Endocrinology 1987; 121:1447-1453.

Stricker P, Grueter R. Action du lobe antérieur de l'hypophyse sur la montée laiteuse. C R Soc Biol 1928; 99:1978–1980.

Suliman AM, Smith TP, Gibney J *et al.* Frequent misdiagnosis and mismanagement of hyperprolactinemic patients before the introduction of macroprlactin screening: application of a new strict laboratory definition of macroprolactinemia. Clin Chem 2003; 49:1504-1509.

Tang X-M, Rossi MJ, Masterson BJ *et al.* Insulin-like growth factor, IGF-I receptors, and IGF binding proteins 1-4 in human uterine tissue: tissue localization and IGF-I action in endometrial stromal and myometrial smooth-muscle cells in vitro. Biol Reprod 1994;50:1113-1125.

Thyresson HN, Su WP. Familial cutaneous leiomyomatosis. J Am Acad Dermatol 1981; 4:430-434.

Tiong TS, Stevenson JL, Herington AC. Regulation of prolactin receptor gene expression by thyroid hormone status in the rat. J Mol Endocrinol 1992; 8:63-72.

Tomlinson IP, Alam NA, Rowan AJ, *et al.* Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet 2002; 30:406-410.

Vallette-Kasic S, Morange-Ramos I, Selim A, *et al.* Macroprolactinemia revisited: a study on 106 patients. J Clin Endocrinol Metab 2002; 87:581–588.

Velebil P, Wingo PA, Xia Z, *et al.* Rate of hospitalization for gynecologic disorders among reproductive-age women in the United States. Obstet Gynecol 1995; 86:764-769.

Vogelstein B, Fearon ER, Hamilton S, *et al.* Use of restriction fragment length polymorphisms to determine the clonal origin of human tumors. Science 1985; 227:642-645.

Wack JT, Rodin J. Smoking and its effects on body weight and the systems of caloric regulation. Am J Clin Nutr 1982 ; 35:366-380.

Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. Embo J 1994; 13:2182-91.

Walker AM, Montgomery DW, Saraiya S, *et al.* Prolactin-immunoglobulin G complexes from human serum act as costimulatory ligands causing proliferation of malignant B lymphocytes. Proc Natl Acad Sci USA 1995; 92:3278–3282:246–249.

Walker SE, Allen SH, McMurray RW. Prolactin and autoimmune disease. Trends Endocrinol Metab 1993; 4:147–151.

Walters CA, Daly DC, Chapitis J, *et al.* Human myometrium: a new potential source of prolactin. Am J Obstet Gynecol 1983; 147:639-644.

Weitzman RE, Leake RD, Rubin RT, *et al.* The effect of nursing on neurohypophyseal hormone and prolactin secretion in human subjects. J ClinEndocrinol Metab 1980; 51:836-9.

Whittaker P, Wilcox T, Lind T. Maintained fertility in a patient with hyperprolactinemia due to big big PRL. J Clin Endocrinol Metab 1981; 53:863–866.

Wilcox LS, Koonin LM, Pokras R, *et al.* Hysterectomy in the United States, 1988-1990. Obstet Gynecol 1994; 83:549-555.

Xu, GY, Yu HA, Hong J, *et al.* Solution structure of recombinant human interleukin-6. J. Mol.Biol. 1997 ; 268, 468–481.

Yoshimura Y, Hosoi Y, Iritani A, *et al.* Development potential of rabbit oocyte matured in vitro: the possible contribution of prolactin. Biol. Reprod 1989.; 41: 26-33.

Zink T, Ross A, Luers K, *et al.* Structure and dynamics of the human granulocyte-colony-stimulating factor determined by NMR-spectroscopy-loop mobility in a 4-helix-bundle protein. Biochemistry 1994; 33: 8453–8463.

Zhu YL, Conway-Campbell B, Waters MJ, *et al.* . Prolonged retention after aggregation into secretory granules of human R183H-growth hormone (GH), a mutant that causes autosomal dominant GH deficiency type II. Endocrinology 2002; 143: 4243–4248.

Appendices

No.: Name: Hospital No.: Date: Birth date:

Age of menarche:
Chief complain:
M.C.= *Pain:* NO ☐ Yes ☐ *Amount:* Heavy ☐ Normal ☐

LMP:

Single Yes ☐ NO ☐ Marriage date:

Chief complain:Pregnant Yes ☐ NO ☐ G ◯ P ◯ A ◯

1st pregnancy date: Last birth date: Last abortion date:

Infertility NO ☐ Yes ☐ *Primary* ☐ *Secondary:* ☐

Drug History: NO ☐ Yes ☐

Current contraception:NO ☐ Yes ☐ OCP ☐ Inj. ☐ Others ☐

Previous contraception: NO ☐ Yes ☐

Smoking: NO ☐ Yes ☐ No. of cigarette: Duration:

Wt.(Kg): Ht.(m): BMI(Kg/m^2)=

S.urea(mg/dl): S.creatinin(mg/dl): S.TSH: $S.T_4$: S.Total protein(gm/dl):
S.albumin(gm/dl):

Presentation:
No symptoms ☐ V.B ☐ Mass ☐ Pressure ☐ Pain ☐ Pregn.loss ☐

Infertility ☐ Others ☐

The uls examination of LM:

LM operation Hysterectomy Myomectomy
Date Indication Dr.(operation) Duration of sampling:

Total No. of fibroid in uterus: **Diameter(cm)** **Depth** **Site**

No.1:
No.2:
No.3:
No.4:
No.5:
No.6:

S.PRL(ng/ml): Pituitary MRI report:

Endometrium (Histopathological report):

Please fill all the areas and mail the saved form (in word format) along with your thesis/ dissertation to journalijar@gmail.com.