

Trimegestone
and
The Endometrium

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Trimegestone and the Endometrium

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Abstract

The health benefits of long term postmenopausal HRT are well recognised, but the re-initiation of cyclical bleeding, especially when irregular or heavy, has a profound impact on continuation rates. In this thesis, this clinical problem was investigated in 256 postmenopausal women who participated in a randomised dose ranging study of sequentially administered trimegestone (4 doses) with continuous micronised oestradiol, for 6 months.

A strong dose dependent modulation of the bleeding pattern was documented. Women in the higher trimegestone dose groups had better bleeding pattern compared to those on the lower doses. This was subsequently confirmed when 134 women completed a further 6 months' extension study with a single dose of trimegestone (0.25 mg). The presence of submucous fibroids in 176 women predicted the occurrence of abnormal bleeding pattern, however, the dose of trimegestone remained the dominant factor.

These clinical data led to detailed histological and immunohistochemical evaluation of the endometrial samples collected on day 24 of the last treatment cycle. Histomorphometric parameters, differential leukocytic population, the microvasculature and cell proliferation and apoptosis markers were studied.

The majority of the results showed remarkable similarities between the test endometria and the natural cycle. None of these parameters clearly demonstrated a dose-dependent effect of trimegestone. When the expression of metalloproteinases -1 and -3 were assessed in specimens from women who received the highest and the lowest dose of trimegestone, where the bleeding pattern was completely different, no dose effect was demonstrated.

These conclusions led me to question the ability to demonstrate this apparent similarity of endometrial effect between trimegestone and progesterone. For this I have developed a primary stromal cell culture system, and examined the expression of MMP-1, and -3 mRNA in these cells. Similarity between trimegestone and progesterone was again demonstrated.

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The laboratory work in this thesis involved:

1. *immunohistochemistry* : I have developed and completed all optimisation experiments for all immunohistochemical stains in this thesis. Subsequent staining was performed by the laboratory technical staff in the Department of Obstetrics and Gynaecology.
2. *Image analysis* : I am totally responsible for the capture of about 32 000 images described in this thesis and their subsequent morphometric analysis
3. *Primary stromal cell culture* : I have established this technique and optimised the conditions for extended primary stromal cell culture system. I have designed and performed all the experiments mentioned in this thesis.
4. *Molecular techniques* : I attended the practical part of the course in Molecular Toxicology at the Department of Pathology, Leicester University in 1998 and since then. I started the deployment of these techniques in my cell culture system. I have optimised and performed all experiments related to RNA extraction, RT-PCR, agarose gel separation and densitometric analysis of the discernable products. The mRNA isolated in experiments 3 were analysed by the technical staff in the Department of Pathology.

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Common abbreviations used in the text

AR	Androgen receptor
ER	Oestrogen receptor
PR	Progesterone receptor
PAB	Progestogen associated bleeding
TBS	Total bleeding score
IMB	Intermenstrual bleeding
SMF	Submucous Fibroid
NET	Norethisterone
MPA	Medroxy progesterone acetate
LNG	Levonorgestrel
HRT	Hormone Replacement Therapy
P	Proliferative phase
EL	Early luteal phase
ML	Mid luteal phase
LL	Late luteal phase
M	Menstrual phase
H&E	Haematoxylin and Eosin
RT	Room Temperature
MMP	Metalloproteinase
FSH	Follicular Stimulating Hormone
SD	Standard deviation
LDA	Linear discriminant analysis
LDF	Linear discriminant function

Chapter One

Introduction

Women's health issues influenced by the menopause have moved from a peripheral medical interest into a central health care programme that commands a different strategy for resource allocation of research and service delivery. By the turn of the 20th century life expectancy of women in the western world was about 58 years, at present it is about 82, and as the mean age of the menopause (51 years), has not changed; more than one third of a woman's life is expected beyond the menopause. Cessation of menstruation for 12 months defines the menopause in most women ¹, however, more women of a younger age are becoming menopausal, following hysterectomy and bilateral salpingo-oophorectomy for benign reasons such as menorrhagia, endometriosis, and pre-menstrual tension syndrome.

Pathophysiology of the Menopause

The size of the oocyte reserve is a major determinant of the transition to the menopause ². The reduced fertility in the older women is related to the age-associated deficiency in oocyte maturation process, while the endometrium reserves its ability to respond to hormonal changes ^{3 4}. FSH and inhibin-B have both been proposed as indicators of ovarian ageing ⁵. Serum FSH levels remain low till about the age of 43, and then start to rise, while serum inhibin-B levels progressively decline with increasing age. Serum oestradiol levels start to decline from the age of 38 onwards ^{6 7}

In an interesting study, which combined data from other reports, the ovarian content of primordial follicles appears to undergo a sharp decline at about the age of 37 years^{8,9}. Had this rapid decline in the number of primordial follicles not occurred, menstruation might have continued until the age of 70.

The source of oestradiol and inhibin B is the granulosa cell population which lines the growing primordial follicles. As the number of gonadotrophin-responsive primordial follicles decline, and consequently the number of growing granulosa cells, symptoms and signs of oestrogen deficiency start to develop, although the dramatic biochemical and tissue structural changes in women appear only after the menstrual periods have ceased completely. Due to the hypergonadotrophic state, steroidogenesis continues in the ovarian stroma and theca cells, with an increased production of androgens, particularly androstendione and testosterone, and to a lesser extent dehydroepiandrosterone (DHEA), which is peripherally converted into oestrone, a weak but predominant circulating oestrogen in the post-menopause.

Consequences of oestrogen deficiency

Vasomotor symptoms

The hot flush results from spontaneous vasodilatation of skin capillaries, which affects the chest area and spread upwards to facial skin; resulting in an increase in skin temperature by about 1-2⁰ C. Hot flushes usually last for less than a minute and are followed by perspiration. About 58-93% of postmenopausal women suffer from hot flushes, especially in first 2 years ¹⁰. However, the mechanism underlying hot flushes is not clearly understood.

Psychological Symptoms

There is an increased incidence of depression in peri- and post-menopausal women, which affects about 65% of women, and probably, the incidence is higher in women undergoing surgical menopause. Oestrogen can affect the concentration of neurotransmitters in the brain. It increases the rate of degradation of monoamine oxidase (MAO), which catabolises serotonin, whose deficiency is associated with depression and insomnia ¹¹.

There is an increase in incidence of Alzheimer's disease in women after the age of 65 years ¹², and in spite of lack of evidence of direct relation between the oestrogen deficiency and the neurodegenerative process in the brain, oestrogen treatment appears to reduce the incidence of Alzheimer's disease ¹³.

Vulvovaginal atrophy

Oestrogen deficiency is associated with atrophic vulva, which loses its collagen and adipose tissue and become flattened and thin. This may lead to irritation and itching which may interfere with sleep.

Vulvovaginal atrophy is associated with increased incidence of sexual problems, as vaginal dryness may lead to painful intercourse and reduced libido. About 30% of postmenopausal women in one study complained of dyspareunia ¹⁴.

Osteoporosis

The life time risk of osteoporotic fractures for the 50 year old white female is 11% for vertebral fracture, 13% for distal radius fracture, and 14% for femoral neck fracture ¹⁵. Therefore, about one third of women will sustain one or more osteoporotic fractures in their lifetime. The maximum bone mass is attained at the age of 30 years, followed by a plateau for about 10 years, thereafter age-related bone loss of about 0.5% per annum ensues ^{16 17}. At the menopause, a phase of accelerated bone loss of about 4% per annum has been recognised, which affects mainly cancellous bones, and lasts for about 4 years ^{17 18}. Thereafter the annual rate of bone loss slows to 1% ¹⁹.

Cardiovascular disease

Ischaemic heart disease (IHD) is the most common cause of death in women over the age of 55, and accounts for about 25% of deaths. The menopause is an independent risk factor for ischaemic heart disease

(IHD). The overall relative risk for IHD in the menopause is 2.7 and this risk rises with younger age at menopause²⁰. In women with a previous myocardial infarction (MI), ever-use of oestrogen significantly reduces age-adjusted mortality from a repeat MI to 0.56 (95% CI 0.50-0.61)²¹. The relative risk for cardiovascular disease in women on oestrogen replacement remains 0.65 even after adjustment for other risk factors such as, previous MI, diabetes, hypertension, smoking, serum cholesterol, BMI, age and education²².

There are structural and functional changes in the heart following, the menopause: reduced stroke volume²³, reduced cardiac output²⁴, left ventricular hypertrophy evident by a 10% increase in posterior wall and septum thickness within 5 years post menopause²⁵, and higher incidence of "Ischaemia Minor" in the ECG's of oophorectomised women²⁶.

A reduction in the incidence of cardiovascular disease including cerebro-vascular accidents, will therefore, have the greatest potential to influence the life expectancy of women.

The lipids factor

The disturbance in lipid metabolism ranks highly among the identified risk factors of atherosclerosis and cardiovascular disease. Elevated serum total cholesterol and triglycerides have been identified to have a linear positive relationship, and high density lipoprotein (HDL-cholesterol) was found to have an inverse relationship with the risk of coronary heart disease.

After the menopause, women retain their higher level of HDL-cholesterol, but this is counteracted by a higher level of low density lipoprotein (LDL-cholesterol), compared to men of the same age. About half the patients with coronary artery disease have reduced HDL-cholesterol levels; the commonest lipoprotein abnormality.

Consequently, elevation of HDL-cholesterol by oestrogen treatment should be viewed as an important vehicle for the cardio-protection effect of this hormone. Oestrogen reduces LDL oxidation and therefore interferes with its uptake by monocytes and tissue macrophages, a particularly important step in the development of atherosclerosis. The significance of raised serum triglyceride, as an independent risk factor for cardio-vascular disease is still a controversy.

About 30-50% of the cardio-protective effects of oestrogens has been attributed to the changes in LDL- and HDL-cholesterol. In addition, oestrogen reduces central obesity, and together both reduce the insulin levels. Indeed, current oestrogen use may reduce the risk of insulin independent diabetes mellitus by 20%²⁷.

Oestrogen deficiency is associated with increase in LDL-cholesterol and reduction in HDL-cholesterol²⁸. Oestrogen replacement reverses these changes. Androgenic progestins may increase the catabolism of HDL-2, with the result of lowering the levels of HDL2 and total HDL-cholesterol²⁹. In addition, oestrogen with cyclically administered

progestogen has little or no effect on triglycerides ³⁰. When a progestogen is used in a continuous combined fashion along with oestrogen, as an amenorrhoea regimen, HDL and triglyceride levels were reduced ³¹.

The HERS Study

The Heart Estrogen/progestin Replacement Study (HERS) was a randomised placebo controlled study of continuous combined conjugated oestrogens (0.625 mg) plus 2.5 mg of medroxyprogesterone acetate (MPA) for the secondary prevention of ischaemic heart disease (IHD) in women with established diagnosis of IHD ³². In contrast to the investigators expectation there was an increase of IHD events in the treated group compared to the placebo group during the first two years of the study. However, when the first two years were free from inter current IHD, there was a trend towards a beneficial effect in the treated group ³³.

This trial was a landmark in this field but unfortunately suffered from a number of pitfalls, which resulted in more questions than it could answer. The recruitment period was extended with the majority of patients were enrolled towards the end of the 18 months period, and the observation time was thus reduced to a mean of 4.1 as opposed to 4.75 years in the initial description of the protocol. More importantly, the choice of the progestogen may have affected the results. The administration of MPA to oestrogen primed ovariectomised rhesus

monkeys induced more sustained coronary vasospasm, and in one animal out of six a fatal response to the serotonin/acetylcholine intracardiac infusion did occur, while progesterone administration in the control set did not affect coronary artery vasoactivity³⁴.

Hormone replacement Therapy

It is clear that oestrogen deficiency associated with the menopause, endows the women with a host of symptoms, which degrade her quality of life, and induces a series of biochemical changes which make her susceptible to cardiovascular disease and osteoporotic fractures. Oestrogen replacement treatment improves quality of life, and in the majority of women reverses those adverse biochemical changes. The effectiveness of such treatment, nevertheless, is manifest in many epidemiological data on the health benefits of such treatment, albeit that almost all of these studies have borne one or more stigma of bias or less than optimal methodology. Despite all reservations, it is generally agreed that the long term health promotion effect of oestrogen in postmenopausal women on disability-free life expectancy or on all cause mortality, is a function of duration of oestrogen use. Nevertheless, the reported increased incidence of breast cancer with long term oestrogen replacement and the re-establishment of menstruation rank highly among the reasons for early discontinuation of postmenopausal hormonal supplementation.

Oestrogen

The three main oestrogens detected in circulation are oestradiol, oestrone and oestriol. Oestradiol has the highest biological activity.

Oxidation of the hydroxyl group at C-17 of oestradiol gives rise to oestrone, which shows about 50-70% the activity of oestradiol ³⁵.

Further addition of hydroxyl group at C-16 results in oestriol which has about one tenth the activity of oestradiol ³⁵. A number of synthetic

oestrogens have been synthesised in order to enhance its absorption such as, ethinyloestradiol (EE), by the insertion of ethinyl group at C-17.

EE is highly water soluble, very potent oestrogen since it is resistant to intracellular 17β dehydrogenase, and therefore has a more sustained

activity. It is widely used in oral contraceptives, but is not used for postmenopausal HRT. About 60% and 40% of oestradiol are bound to albumin and SHBG, respectively, with only 2% unbound. Oestradiol is

readily converted to oestrone through the enzyme 17β hydroxysteroid oxido-reductase (dehydrogenase). Metabolites of oestradiol and oestrone circulate as sulphates, glucuronides, or mixed conjugates.

In postmenopausal woman, oestrone is the predominant oestrogen. Its primary source is the aromatization of androstenedione (95% from the

adrenals and 5% from the ovaries). There is an increase conversion

with increasing age and weight. The primary source of oestradiol in

postmenopausal women is the peripheral conversion of oestrone by

the enzyme 17β hydroxysteroid dehydrogenase ³⁶.

Measurement of oestrogen potency

The purpose of pharmacological assays is to assess the clinical effect of the drug, regarding efficacy and adverse effect. Potency is the amount of drug necessary to produce a specified effect; a drug which produces an effect at a low dose is more potent than another drug that requires a higher dose to produce the same effect.

Oestrogen induces keratinization of the vaginal epithelium of spayed female rodents. The presence or absence of leukocytes in the vaginal smear provides one end point for assessment of oestrogen potency.

Alternatively, as oestrogen induces uterine growth in rodents (mouse and rat) due to increased in water retention by its tissue; weighing of the organ has been used to bioassay oestrogen effect ³⁷.

Clinical aspects of oestrogen use

Oestrogen is administered orally or parenterally in the forms of injectables, per vaginam (cream, tablet or silastic ring), subcutaneous pellets, intranasal spray, and transdermal (adhesive patches and gel).

All oral preparations result in higher oestrone levels than oestradiol, because oestradiol is metabolised by the intestine and liver during the first hepatic passage, to oestrone. Parenteral administration of oestradiol by-passes intestinal-hepatic first-pass metabolism, and therefore results in higher levels of oestradiol than oestrone.

First-pass effect and enterohepatic recycling

Sequestration of oestrogen by the liver results in reduced bioavailability. Comparison of oral and intravenous administration of EE at doses of 50 µg or 100 µg results in bioavailability of 20% to 65%, which clearly accounts for the substantial variation between individuals in serum levels after oral administration ³⁸.

The need for progesterone

Oestrogen replacement should not be administered unopposed in women with intact uterus, as it may stimulate endometrial growth and results in a high incidence of endometrial hyperplasia and a high risk of developing endometrial carcinoma, an effect which persists even 5 or more years after cessation of treatment ^{39 40 41}. It has been established that the administration of a progestogen for 10-12 days every 28 days may protect the endometrium against hyperplasia and may reduce the risk for developing endometrial carcinoma. NET administered in 1mg dose confers secretory changes in the endometrium similar to 125µg of LNG or 11mg of MPA when given for 7 days in conjunction with oestrogen ⁴². Recent report, however, showed that cyclical sequential combined regimens with progestogens administered for fewer than 16 days was associated with an increase relative risk of endometrial carcinoma OR = 2.9 ⁴¹.

Metabolism of progesterone

Progesterone has a negligible potency when administered orally, and therefore it has to be administered vaginally, rectally or as injectables. This limitation stimulated chemists to search for orally active compounds which have the properties of the natural progesterone on the endometrium³⁸. Norethynodrel, a 19-nor compound was the first orally active progestogen to be synthesised in 1955 by Frank Colton³⁸. Most of the progesterone in circulation is bound to proteins, 20% is bound to corticosteroid-binding globulin (CBG), and the remainder is bound to albumin. Progesterone is metabolised extensively in the liver, and its metabolites are conjugated forming sulphate and glucuronide derivatives, which are excreted, mainly in the urine, but also in faeces. Quantitatively, the most important urinary metabolite of progesterone is pregnandiol glucuronide.

Uterine glandular proliferation model (Clauberg test); has been used to assess progesterone potency. The test involves oestrogen administration to female rabbits, followed by oral or parenteral treatment with the test drug. Progesterone induces the development of complicated glandular structures in the endometrium. There is standardised scale between 0 and 2 to grade the glandular proliferation⁴³. The dose of progesterone that induces glandular changes equivalent to score of 2, signifies an active dose of progesterone. Accordingly, MPA is more potent than LNG, while norethisterone and norethynodrel are weak progestogens⁴⁴.

The Progestogens *Norethisterone (NET)*

The peak serum NET in plasma occurs within 1 to 2 hours of oral administration⁴⁵. However in some women this peak is delayed for up to 4 hours, the reason for this variability may be due to the net effect of the intestinal hepatic first pass. It binds to albumin, but much more strongly to SHBG, and the variation in the first pass effect is responsible for the difference in bioavailability. There is rapid excretion of up to 80% of the dose in the urine mainly within 24 hours of dosing. The urinary metabolites are mainly conjugated to glucuronic acid. However, some of the metabolites are eliminated very slowly, which suggests that with long term administration, there is the possibility of accumulation. Some of NET may be metabolised to ethinyloestradiol⁴⁵.

Levonorgestrel (LNG)

LNG is absorbed rapidly from the intestine, but unlike NET does not undergo first-pass effect, where $87 \pm 9\%$ of the oral dose administered was considered bioavailable as assessed by measurement of the area under the curve⁴⁶. Most of LNG is bound to albumin and SHBG, but it binds to SHBG more avidly than NET. LNG has a very strong anti-oestrogenic activity, and therefore is associated with a lesser increase in SHBG than when other progestogens are administered with oestrogen⁴⁵.

Medroxyprogesterone acetate (MPA)

After oral administration of MPA (10 mg), a peak serum concentration of 1.2 - 5.2/ml is observed within 2 hours. It can still be detectable in

plasma after 72-96 hours. Over 90% of serum MPA are bound to albumin. MPA (150 mg) administered as intramuscular injection, will still be detectable after the end of 90 days interval, and in some women it continues to be detectable up to 200 days. However women who absorb depot MPA quickly from the injection site will have low levels at the end of 90 days. Unlike NET, MPA excretion in the bile and faeces is greater than its urinary excretion.

Trimegestone

Trimegestone (RU 27987) $C_{22}H_{30}O_3$, 17β -{(S)-2-hydroxypropanoyl}-17-methyl-estra-4,9-dien-3-one, is a novel norpregnane progestin.

Studies of the interaction of trimegestone with human recombinant receptors showed a 6 folds stronger relative binding affinity (RBA) for the progesterone receptor than progesterone. Trimegestone had a 40 times lower RBA than testosterone for the androgen receptor, 8 times lower than dexamethasone for the glucocorticoid receptor, and 2.5 times lower RBA than aldosterone for the mineralocorticoid receptor.

Trimegestone is devoid of any affinity for the oestrogen receptor α .

These data suggest that trimegestone has a high specificity for the progesterone receptor (PR), with no significant affinity for other steroid receptors.

Given orally, trimegestone is about 25 - 60 times more active than MPA and NET, while the administration of trimegestone through the subcutaneous route, it is 6 and 1000 times more active than MPA and

NET, respectively, in inducing uterine transformation, according to McPhail scale ⁴³⁻⁴⁷. The RBA of trimegestone was compared to those of the reference agents on human recombinant steroid receptors, MPA, NET, gestodene and LNG (Table 1. 1).

The number of receptor binding sites is similar for both trimegestone and progesterone and the dissociation rate of trimegestone is about 10 times slower than that of progesterone.

Trimegestone is rapidly absorbed after oral administration with C_{max} being reached after 30 minutes. It is eliminated predominantly by the faecal route. Given orally, trimegestone, causes a dose-dependent increase in endometrial transformation in the rabbit, as described by McPhail scale (1934) ⁴³, like MPA and NET.

Mifipristone (RU486)

Mifipristone is a steroid derived from NET. The peak plasma concentration after oral administration is obtained after 1.3 hours, with bioavailability of about 70% ⁴⁸. About 98% are bound to plasma proteins, mainly α 1-acid glycoprotein. RU486 acts as a powerful antagonist of progesterone or glucocorticoids ⁴⁹. It binds to PR with a higher affinity than progesterone, and it binds to both PR_A and PR_B receptors. Oral administration induces interruption of the luteal phase and results in termination of early pregnancy ⁵⁰.

The ability of the progestogens to induce secretory changes in the endometrium was taken as an indicator of the adequacy of the HRT regimen in protecting the endometrium against excessive proliferation. Studies on the histology of the endometrium under the effect of HRT has been compared to that of the natural cycle ⁵¹.

Histology of the endometrium

Endometrial healing following menstruation is usually complete by day 5 post menstruum. This is followed by a phase of growth under the effect of oestrogen, which, quantitatively, dominates the endocrine milieu until ovulation.

The proliferative phase

In the early proliferative phase (day 4-7), the glands are straight and narrow, evenly distributed in a loose stroma and the surface epithelium is flat. The stromal cells have small dense nuclei in scanty cytoplasm. In the mid proliferative phase (day 8-11) there is an increase glandular tortuosity and glandular epithelial mitoses. The surface epithelium is low columnar. In the late proliferative phase (day 12-14), there is marked tortuosity of the glands, with pseudostratification appearance of the lining epithelium, and prominent mitoses. The surface epithelium becomes columnar, and the stromal cells exhibit frequent mitoses ^{52 53}. There are difficulties in dating the endometrium in this phase as early, mid or late proliferative, not only is it due to the

overlap of the histological characteristics, but is also due to the inter-observer variations between pathologists ⁵⁴.

The luteal phase

Following ovulation, corpus luteum formation is endowed with steroidogenesis. The main steroids synthesised are the oestradiol and progesterone.

The luteal phase usually lasts for 14 days and histological dating depends on the changes observed in the glandular epithelium during the first week following ovulation, such as, mitoses , pseudostratification, subnuclear vacuolation, which then become translocated to supranuclear position, and finally intra-glandular secretion. Changes in the stroma characterise the second week following ovulation and are mainly those of stromal oedema, mitoses, predecidual reaction, and leucocyte infiltration ^{52 53 55}.

These changes start 36-48 hours following ovulation. Basal vacuoles appear in the glandular epithelium on the second day and the epithelial nuclei form a single row. A small rim of mucopolysaccharides can be observed at the luminal margin of the epithelial cells on the 4th day. On the 5th day the majority of the nuclei have returned to the base of the glandular epithelium. On the 7th day the glandular lumen is filled with glycogen and there is patchy development of stromal oedema which reaches its maximum on the 8th day. On the 9th day, stromal oedema starts to subside and glandular secretion ceases.

By the 10th day postovulation, the endometrium shows two distinct layers, the upper, a compact layer, which consists mainly of stromal cells, where predecidual changes develops, and a lower layer of distended tortuous glands. Endometrial thickness starts to regress by the 12th day. T lymphocyte, mast cells and polymorphonuclear granulocytes appear in the premenstrual endometrium ⁵³.

The menstrual phase

On days 13 and 14 postovulation, the endometrium show evidence of dissociation of structure of glands and stroma, together with patchy haemorrhages in the superficial layer, while the basal parts remain intact ⁵³.

Hormone Replacement Therapy and withdrawal bleeding

The compromised quality of life as a result of the menopause can be easily improved by oestrogen replacement. Over the last 40 years hormone replacement therapy (HRT) has been increasingly used by women, however compliance remains the main obstacle for women to gain the long term benefits of HRT. After 1 year of HRT, 54.4 % were non-compliant with the treatment ⁵⁶.

The addition of a progestogen in a cyclical manner results in the re-initiation of cyclical bleeding, and for the majority of postmenopausal women is not a welcomed event. One of the main causes of non-compliance with HRT in peri- and post-menopausal women is withdrawal bleeding, particularly if irregular or heavy ^{57 58}. About 44%

to 50% of women discontinue their treatment because of bleeding problems^{59 60}, while in 50% of instances such bleeding occurs before the end of the progestogen phase of treatment⁶¹.

Cyclical sequential HRT regimen, attempts to mimic the effect of the natural cycle on the endometrium where oestrogen is administered continuously with the progestogen added in the last 12 or 14 days of the cycle. The composition of each regimen is designed to produce a good cycle control, to reduce the incidence of hyperplasia, and at the same time is associated with minimal progestogen related adverse effects. In one study, about 77-81 % of women on cyclical sequential conjugated oestrogens and 5 or 10mg of MPA for 14 days, had regular withdrawal bleeding⁶². However, many women were not happy to cope with the withdrawal bleeding after probably years of amenorrhoea.

A fixed dose of oestrogen and a progestogen are used in the so called continuous combined HRT (ccHRT), intended to induce amenorrhoea. The continuous administration of a progestogen offsets the growth stimulatory effect of oestrogen and therefore the induction of thin and atrophic endometrium as documented hysteroscopically and histologically⁶³.

In a double blind study, two amenorrhoea regimens were compared, and women were given either, ccHRT in the form of micronised oestradiol valerate 2mg and norethisterone 0.7 mg daily, or tibolone 2.5 mg/day. Women treated with this ccHRT had a higher incidence of heavier and prolonged bleeding episodes in the first reference period

compared to the women who received tibolone, but at the end of one year the percentage of women with amenorrhoea was similar in both groups ³¹. However there were still women who continued to bleed even after 6 months of treatment. That is in addition to the fact that continuous administration of the progestogen is associated with a higher incidence of premenstrual type of symptoms such as bloatedness, fluid retention and headaches. However, a number of clinical investigators reported that such symptoms were suppressed as a result of the continuous administration of the progestogen ^{64 65}.

Data Collection

Owing to problems of the ability of women to recall menstrual dates and patterns, as collected through interviews or questionnaires, such data are less accurate the longer the interval is between the occurrence of the event and its reporting. Therefore, for more accurate data collection, prospective recording is recommended and is best achieved by conscientious marking of bleeding episodes or spotting (blood loss that does not require sanitary protection) in a menstrual diary, as well as the marking of the bleed-free days.

In analysing menstrual diaries problems are encountered as to whether to include spotting episodes or the truncated events of bleeding or spotting that occur before or after one or two days from the menstrual event. Statistically presented summaries of menstrual data particularly those collected over long observation periods may not be

seriously affected by such episodes in naturally menstruating women who are not using contraceptives. In these women they are largely accepted as part of the physiological phenomenon.

Nevertheless, the acceptability of exogenous sex steroids therapy for contraception or for HRT, is affected by the withdrawal bleeding it creates. This involves regularity of day of onset, duration and severity of bleeding, and the occurrence of inter-menstrual bleeding. In women using intrauterine contraceptive device (IUCD), oral contraceptive pill, or HRT such events may cause inconvenience or embarrassment to the women, who become more conscious of their occurrence and therefore these events have to be included in these analyses. The occurrence of spotting, intermenstrual bleeding, and of truncated bleeding events may influence the acceptance of the treatment being administered, and equally importantly as a phenomenon of altered endometrial behaviour they deserve more detailed examination. Indeed, in the presence of hormonal treatment these issues become important for the postmenopausal woman, where the exclusion of endometrial neoplasia is required, and as it frequently happens that an individualised therapeutic approach to the pattern of bleeding is necessary, in order to enhance the continuation of treatment.

Analysis of the bleeding data

The understanding of the bleeding phenomenon and the ability to predict which woman is likely to bleed irregularly may enhance the clinical management of these women and devise an alternative approach to enable them to continue with the treatment long term.

An in-depth understanding of the bleeding phenomenon may help to provide an opportunity for a scientific approach to endometria, which respond poorly to a particular HRT regimen.

In clinical studies involving postmenopausal women treated with a cyclical sequential combined HRT regimen, for example, the summary statistics of mean and standard deviation used for the description of the day of onset of bleeding, its duration, and the amount of blood loss may give an idea as to the overall efficacy of that regimen. However, summary statistics tend to be dominated by data obtained from women with frequent short cycles. The problem of isolated episodes of bleeding between menses (inter-menstrual bleeding) and the problem of truncated events whether spotting or frank bleeding may complicate the summary statistics. However, the omission of these events from the analysis may overlook substantial factors for discontinuation of treatment and influence acceptability. Moreover, such presentation of data ignores the individual's woman's experience of the bleeding events ⁶⁶.

Analysis of menstrual data may be based on the conventional perception of 28-day menstrual cycles, and this is useful when assessing menstrual rhythm in conjunction with a 28-day HRT regimen. A reference period method of observation based on multiples of 30 days has been suggested ⁶⁷, since menstruation occurs as a “monthly event”. The reference period method is particularly useful when assessing bleeding events associated with IUCD, long acting injectables contraceptive steroids and with progestogen only pill. As such, this method assesses the bleeding events as they happen during the observation period. Regardless of the length of the reference period, be that a multiple of a 28- day cycle, 30 or 90 days, it has to start with the commencement of treatment which allows uniformity of assessment.

Therefore, it is recommended to analyse the individual woman’s diary collected during a treatment phase in order to adjust the dose of treatment or mode of administration of the HRT regimens. A menstrual diary completed by an individual woman allows to present individual responses to a particular treatment without forcing the data into an artificial method of analysis to which neither the woman nor the clinician is able to relate. Habiba et al. (1996) ⁶¹, assessed the bleeding pattern of the individual woman in a group of sequential combined NET-based HRT-treated postmenopausal women. Two types of bleeding pattern were recognised. Early bleeders who bled before the completion of the progestogen phase, had a wide variability of the mean day of onset of the progestogen associated bleeding, heavier and

prolonged bleeding episodes. The second group described as “late bleeders”, who bled after the completion of the progestogen phase, whose cycles were less variable, shorter and lighter. This simple subdivision of menstrual behaviour on HRT may help women to adjust their life style, with some degree of precision.

Effect of exogenous sex steroids on the bleeding pattern in premenopausal women

The perceived belief that the natural cycle is a regular 28-day event is not true. In the first half of the 20th century numerous publications confirmed that in the general population cycle length, and the duration of bleeding are variable not only between women but also between cycles in the individual woman as well. Belsey et al. (1991) ⁶⁸ reported that the median number of bleeding days is 19 in the 90 days references period. Fifty percent of naturally menstruating women has at least 10 days difference between the longest and shortest bleeding segments per year, and 50% of women has an episode which lasts at least one week or more. These findings agreed with Trelor et al. (1970), who had reported that the average cycle length was 25 to 41 days ⁶⁹.

Breakthrough bleeding associated with oral contraceptives has been one of the reason for low compliance with this type of contraception. Women in this young age group may sometimes accept irregularity of bleeding including intermenstrual bleeding for the benefit of contraception, nevertheless, postmenopausal women who use HRT to improve their quality of life, are less tolerant to bleeding irregularities.

Effect of route of administration of progestogens

The route of administration of progestogens plays a significant role in the modulation of bleeding pattern. In a study by Belsey et al. (1991)⁶⁸, 6 different sex steroids were given for 12 months to young women requiring contraception (Table 1. 2).

Sex steroids administered were: the combined oral contraceptive pills containing ethinylloestradiol (EE) 50µg and 0.25mg LNG, EE 50µg and 1mg of NET, a monthly combined injectable containing oestradiol cyprionate 5mg and MPA 25 mg, oestradiol valerate 5mg and norethisterone enanthate 50 mg, a vaginal ring releasing 20µg LNG daily, or long acting injectable depot MPA (DMPA) given every 3 months⁶⁸ for 12 months.

The median number of bleeding and spotting days in the 90 days reference period were 13.5, 15.6 and 17.5 days in the combined pills, monthly injectable and vaginal rings respectively, while DMPA was associated with least bleeding days. Women using DMPA had the longest median bleeding-free intervals, of 27.4 days. Women using the vaginal rings had the shortest mean bleeding-free intervals of 20.6 days. Median cycle length was 27.8 days in women on the combined contraceptive pills, who had little variability in their cycle length compared to other groups. Women using the vaginal rings had the shortest median cycle lengths of 25.8 days while women on DMPA had the longest median cycle length of 35.9 days.

The use of injectable progestogens is also associated with bleeding irregularities. In a multicentre study, the effect on the pattern of bleeding of DMPA 25 mg with oestradiol cypionate, 5 mg was compared to norethisterone enantate 50 mg with oestradiol valerate 5 mg. Acceptable vaginal bleeding was defined as 3-5 bleeding/spotting episodes, none was longer than 14 days, and the women experienced at least 3 bleeding/spotting-free intervals of 14 days or more. Treatments were given every 30 days as intra muscular (IM) injection for 12 months. Using the reference period method ⁶⁷, there was no major difference in the bleeding pattern between the 2 groups, however, only two third of women in each group fulfilled the definition of acceptable bleeding pattern 65.9% and 65.4%, respectively ⁷⁰.

However, different results were reported with the oral preparations. In a study of 6 different combined oral contraceptives included, EE (2µg, 35µg or 50µg) or mestranol (50µg) in combination with either LNG (150µg) or NET (400µg or 1 mg). Women on preparation containing the lowest dose of EE i.e. 20µg and that containing 400µg of NET continued to experience abnormal bleeding pattern throughout the study period of 24 months, and hence, had a higher rate of discontinuation. This might be due to sub-optimal doses of oestrogen or progestogens and/or altered ratio of these sex steroids required to observe the least episodes of abnormal bleeding ⁷¹.

Effect of dose of progestogen

Li et al. (1992)⁷² studied the effect of the dose of intramuscular progesterone (25-50mg) administered from day 15-28, and oestradiol valerate in a cyclical sequential regimen on the endometrial morphometry in 2 treatment cycles, where women were crossed over to receive either a lower doses of progesterone than the standard dose of 25-50mg i.e. 5-10 mg or higher doses of 125-250 mg IM, after one treatment cycle. All women were treated with 2mg Cycloprognova (Shering, Sussex) for one cycle before the start of the study. Reducing the dose of progesterone to 1/5th the standard dose was associated with higher endometrial glandular mitotic activity, reduced subnuclear and supranuclear vacuolation and an increase in the volume of glands compared to the lower doses of progesterone. They concluded that the advancement of the endometrial development is mediated by mechanisms other than the dose of progesterone.

The same group reported another study, where 2-4 mg of oral oestradiol valerate together with progesterone in a cyclical sequential regimen was sufficient for endometrial development in women with premature ovarian failure. The 1mg of oestradiol valerate however, induced a less mature endometrium, as there was significant reduction in the number of supra- and sub- nuclear vacuolation in the glandular cells, with significantly lower secretory activity in the glands and an increase in glandular volume⁷³.

Oestrogens and progestogens have been used to modify the unscheduled bleeding. The administration of EE in a dose of 50µg/day or LNG 0.3 mg twice daily, for 20 days to women who experienced about 100 bleeding/spotting days in the first year of LNG implants (Norplant) use, resulted in reduction of the number of bleeding/spotting days ⁷⁴. The effect, however was lower in the oral LNG treated group compared to those treated with EE.

It is believed that the incidence of abnormal bleeding patterns will decline with the long term use of sex steroids. In an interesting study published by the World Health Organisation WHO (1987), the bleeding pattern associated with two doses of DMPA of 100mg and 150 mg given every 90 days, was compared ⁷⁵. This report demonstrated a time dependent trend where there was a reduction in the number of bleeding/spotting days, and number and length of such episodes with increased duration of treatment. The only difference between the two doses was a higher incidence of amenorrhoea in the 150 mg dose group.

The structure of steroid receptors

The mechanism involved in the action of a steroid hormone includes the diffusion of the hormone across the cell membrane to the nucleus and binding to the receptor protein. This leads to a reaction between the hormone receptor complex and nuclear DNA. Consequently, messenger RNA (RNA) is synthesised and transported to the

ribosomes in the cytoplasm, leading to protein synthesis, which results in a specific activity. Binding of the hormone to the receptor leads to transformation of the receptor which refers to conformational changes of the hormone-receptor complex, revealing a site in the complex to bind to the chromatin ⁷⁶. When unbound, the receptor is chaperoned by a heat shock protein (HSP). Hormone binding leads to dissociation of receptor-HSP complex. The concentration of oestrogen receptor (ER) is 100 to 1000 folds lower than that of HSP90. Binding of HSP90 to steroid receptor has two functions; in the absence of ligand, HSP90 stabilises the receptor, so it is resistant to chemicals and high temperatures. Binding of HSP90 provides repression of receptor activity in the absence of the ligands ⁷⁷.

Clinically the duration of exposure to a hormone is more important than dose. A major factor in the difference in the potency among various oestrogens is the length of time a particular oestrogen occupies the receptor complex.

Oestrogen receptor has a high turnover following gene activation, which suggest a need for continuous oestrogen administration for continuing response. The receptors exit continuously from the nucleus to the cytoplasm and then back to the nucleus upon further ligand binding.

Oestriol has 20-30% the affinity to ER than oestradiol, and therefore it is rapidly cleared from the cell, however if the effective concentration is equivalent to oestradiol, then it can produce a similar biological

activity ⁷⁶. Alternatively, the higher rate of dissociation from the receptor can be compensated for by continuous application of oestriol to allow prolonged nuclear binding.

ER contain 2 regions required for transcriptional activity;

Transactivating Function-1 (TAF-1), which is located near the amino terminus of ER and TAF-2 which is located near the carboxyl terminus.

Wild type ER responsiveness require both TAF-1 and TAF-2 activity, however, TAF-1 and TAF-2 can manifest their respective activity independently.

Oestrogen does not only induce transcription through ligand binding, but also induces common intracellular pathways in different cells.

There is a link between ER on the cell membrane and the mitogen-activated protein (MAP) kinase signaling cascade ⁷⁸, MAP kinases are family of serine and threonine kinases which under the influence of growth factors, become phosphorylated and activated. Example of this pathway is the 17 β oestradiol-induced rapid activation of nitric oxide synthase (sNOS) in pulmonary artery endothelial cells. Similarly the MAP kinase pathway is activated in MCF-7 cells under the effect of ER complex ⁷⁹.

The distribution of ER and PR in the endometrium

Oestrogen treatment of the endometrium increases endometrial PR expression. Indeed, PR expression is a marker of full activation of the ER. Progesterone treatment actually down-regulates PR expression.

expression^{80 81 82 83}. Since the generation of the ERKO (oestrogen receptor α knockout mice), the effect of the other forms of ER i.e. ER β was studied⁸⁴. ER α and ER β are not always expressed in the same cell, suggesting that some oestrogen actions may be mediated via ER β and not ER α . The highest expression of ER β is in the ovary, uterus and the breast⁸⁵. Both receptors, however, are expressed in the endometrium, in the glands and stroma and in all the phases, though with a weaker expression in the secretory phase⁸⁶.

PR on the other hand, exists in 2 isoforms, PR_A and PR_B. The transcription of both PRs is controlled by different promoters that are under the control of oestrogen^{87 88}. The concentration of PR in the endometrial glands in the endometrium of the natural cycle is low in the menstrual and early proliferative phase, but starts to increase in the mid and late proliferative phases^{89 90 91}, while it is low in the luteal phase, when serum progesterone is elevated. In the stroma it is steady throughout the natural cycle^{89, 90 91}. Shiozawa et al. (1996)⁹² found similar changes in the stroma and the glands, where the percentage of cells stained positive to PR and ER in the proliferative phase, started to decrease in the early luteal phase, and then to a low level in the mid and late luteal phase.

Pregnancy associate endometrial, α 2-PEG, Placental protein 14 (PP14), Glycodelin, is an α 2-globulin secreted by the endometrium, and exclusively by the endometrial glandular cells and is regarded a marker

of PR expression in the epithelium. It is undetectable after 5th day of the beginning of the natural cycle, increases in mid and late luteal phases, i.e. around time of implantation, but mainly in the late luteal phase when steroid hormone levels are falling ^{93 94 95}. The highest expression is around day 10 postovulation ⁹⁵. Progesterone is necessary for PP14 production and the endometrium in the anovulatory cycles lacks its expression. Many factors influence the secretion of PP14, such as mifipristone, tamoxifen, GnRH analogue ⁹⁶. Habiba et al. (2000) ⁹⁷ reported that PP14 is expressed in the HRT cycle, but, its levels were lower than the luteal phases of the natural cycle.

Menstruation

The human endometrium is a tissue characterised by cyclical proliferation, differentiation and shedding. Menstrual discharge is a declaration of implantation failure and represents a mechanism for the creation of a new environment for future implantation. Markee (1940) showed that vascular dilatation and subsequent rupture of microvasculature heralded menstruation using endometrial explant in the anterior chamber of the eye of Rhesus monkeys ⁹⁸.

Endometrial shedding involves the functionalis layer ⁹⁹, but spares the basalis, and this process is complete by 60 hours ¹⁰⁰. Regeneration occurs to form a new lining surface from the epithelium preserved in the glandular stumps at the basalis, followed by the growth of stromal and endothelial cells ^{99 101}.

Regeneration starts 24 hours after the onset of bleeding and complete surface reepithelization occurs by day 5 ¹⁰¹.

There have been many studies which attempted to explain the mechanism underlying endometrial shedding of the menstrual cycle. Whether the initiating event involves vascular spasm and subsequent rupture of vascular spaces and capillaries or the disintegration of the extracellular matrix of the endometrium is not very clear. Markee (1940)⁹⁸ observed in his endometrial transplant experiments, that a regression of endometrial thickness by 25-75% and constriction of the spiral arterioles occurred before the onset of bleeding. The spasm of spiral arterioles was followed by vasodilatation and bleeding. He also observed that 75% of the bleeding was of arterial origin, and the bleeding stopped after the spiral arterioles had undergone spasm again.

Progesterone withdrawal bleeding occurs at focal points of the endometrium ¹⁰². Ideally, the study of these foci may provide a clue to the understanding of mechanisms involved in endometrial bleeding and shedding compared to adjacent endometrium. However, the collection of tissue from these sites is not an easy task. Moreover, it is not possible to predict the bleeding area. One way of obtaining a biopsy from a targeted site is by the use of Leicester Endometrial Needle Sampler (LENS) ¹⁰³, using Hyscone to distend the uterus. LENS is safe, easy to use, and each biopsy can provide up to 30 histological sections for assessment.

Structural abnormalities involving the endometrium

In a previous report 47.6% of women with irregular or heavy bleeding on HRT had structural lesions on hysteroscopic assessment ¹⁰⁴. Similar results (46.7%) were reported by Nagele et al. (1996) ¹⁰⁵. Akkad et al. (1995) ¹⁰⁴ found that the presence of submucous fibroids was associated with a three-fold increase in the risk of abnormal uterine bleeding in pre-menopausal women and a two-fold increase in the risk of abnormal withdrawal bleeding in peri- and post-menopausal women using HRT.

Endometrial polyps, while invoked as a cause of abnormal bleeding patterns, previous reports failed to show such a correlation ^{104 106}.

Functional markers in the endometrium relevant to menstruation

Cellular growth and differentiation involves myriad factors which ultimately regulate tissue architecture. These include maintenance of vascular supply, the production of extracellular matrix, factors regulating tissue repair and proliferative signals for cellular replacement; since cells may be lost to senescence or injury. The interplay between these factors in reproductive organs is modulated by sex steroids, the levels of which, change cyclically over a short time during the natural cycle, hallmarked by ovarian follicular development, ovulation and demise of the corpus luteum. These events are represented by endometrial proliferation, differentiation and shedding. From the reproductive biologist point of view, this

tissue furnishes excellent models for the understanding of the various cellular processes involved, as influenced by sex steroids.

In the section to follow, I will briefly review factors identified to be fundamental for maintaining the health and integrity of endometrial tissue and are modulated by sex steroids during the natural cycle.

Proliferation markers

Menstruation is an acute inflammatory process, involving endometrial destruction and shedding of the functionalis layer. This is followed by the process of proliferation where re-epithelisation of the endometrial tissue occurs. In order to understand which component of the endometrium is involved in the healing process and which compartment contributes to the overall growth of this tissue, the distribution of cells actively engaged in cell division are studied.

Ki67

Ki-67 is a non-histone proliferating antigen. It is expressed in all active parts of the cell cycle, but is absent in the resting cell, G0 phase¹⁰⁷. The expression of ki-67 is used to assess proliferation as it does not degrade during apoptosis, however, apoptotic bodies are negative for ki-67 immunostaining^{108 109}. Ki-67 is expressed exclusively in the nuclei of glandular epithelial and stromal cells, in the proliferative phase, then its expression decreases in the early luteal phase before a rise in the mid and late luteal phases of the natural cycle^{110 111}.

This is further explained in Chapter 8.

Cyclins

Cyclins are proteins expressed periodically during progression of the cell cycle. They function by forming complexes with cyclin-dependent kinases (CDK) ⁹². These complexes propel the cell cycle from G1 to S phase.

There are 2 cyclins known to control the cell cycle; G1 cyclin which binds to cdk during G1 and is required for entry into S phase, and mitotic cyclins (mainly cyclin B) which bind to cdc2 during G2 for entry into mitosis ¹¹². The result of this combination is a complex called M phase promoting factor (MPF). As the cell exits the M phase cyclin B1 is destroyed to allow the cell to enter G1 phase ¹¹³. The localisation of cyclins in the cell is different, for example, cyclin A is localised in the nucleus from S phase onward, while cyclin B1 accumulates in the cytoplasm and enters the nucleus only at the beginning of mitosis ¹¹⁴. There is a significant correlation between cdk2 labelling index and the stage of endometrial carcinoma, and between cdk4 labelling index and the pathological stage of the disease ¹¹⁵.

Cyclins may be regulated by sex steroids since they show cyclical changes in the endometrium. Cyclin B1 is expressed in the glands in early proliferative phase, it decreases gradually in the early luteal phase, while its level in the stroma starts to increase in mid and late luteal phases ⁹². cdc2, cdk2 and the other cyclins show cyclical variations with the menstrual cycle.

Extracellular matrix

Integrins

Integrins are a large family of at least 20 members of cell adhesion receptors, which are involved in cell-cell and cell-matrix interaction. They function as transmembrane linker between an extracellular ligand and the cytoskeleton. The structure of integrins consists of 2 subunits; alpha and beta. Each sub-unit contains a large extracellular region which binds to glycoprotein in a calcium dependent manner, a transmembrane moiety and a short cytoplasmic tail which is associated with actin filaments, therefore, integrins may act as transducer ¹¹⁶. The target amino acids sequence for many integrins is Arg-Gly-Asp (RGD) which is common to many ligands such as fibronectins and laminin ¹¹⁷ ¹¹⁸ ¹¹⁹. Integrins expression is upregulated by many factors such as TNF α , TGF- β 1, and IL-1 β ¹²⁰.

Progesterone regulates the expression of α 4 and β 3 subunits as detected by Immunohistochemistry, and this effect is blocked by the anti-progestin, mifipristone. The expression of α v β 3 is influenced by factors other than progesterone such as cytokines, for example epidermal growth factor (EGF), since there is no effect on its distribution in the mifipristone- treated endometrium ¹²¹ ¹²².

There are regional differences in the expression of integrin in the endometrium, viz. the functionalis versus the basalis ¹¹⁷, in addition to differences in localisation at different phases of the menstrual cycle ¹²³.

Aplin et al. (1996) found that in the human endometrium αv is expressed in the apical, lateral and basal epithelium, while $\beta 5$ mainly expressed at apical areas ¹²⁴. $\alpha 5$ is expressed on luminal and glandular epithelium in the proliferative and luteal phases, and decreases in late luteal phase. It is expressed in the stroma and endothelium in the proliferative and luteal phases, but is increased in late luteal phase ¹¹⁶. The low level of $\alpha 5$ at the end of the cycle may allow migration of epithelial cells to repair the endometrium. Some integrins are cell type specific such as $gpIIb/III\alpha$ ($\alpha IIb/\beta 3$) which is expressed only in platelets. Ions such as calcium, magnesium and manganese are essential for ligand binding. $\alpha v\beta 3$ appears abruptly after day 19 of the cycle, and their expression is upregulated by oestrogen and progesterone ¹²⁰. The ability of the endometriotic tissues to express different integrins such as $\alpha 6$ and $\beta 3$ may explain their ability to adhere to peritoneal tissues and recurrence of endometriosis after its remission ¹²⁵.

Matrix metalloproteinases (MMPs)

The endometrial tissue contains collagens, mainly type III, and expresses fibronectin, laminin, which is shed during menstruation. Tissue remodelling requires breakdown and resynthesis of the extracellular matrix (ECM). The matrix metalloproteinases (MMPs) have been implicated in playing a pivotal role in extracellular matrix degradation ²⁴⁰. MMPs are expressed in many tissue

such as uterus, ovary, prostate. A host of MMPs are expressed in the endometrium and been implicated in tissue breakdown at menstruation ^{126 127}. The evidence being gathered, suggest a sex steroid modulation of at least some of the MMPs and may well be involved in the abnormal bleeding associated with sex steroid therapy ¹²⁸. In general, the expression of MMPs declines in the early luteal phase, and reappears again in the late luteal phase. This is further explained in Chapter 11.

Vascular endothelial Growth Factor (VEGF)

VEGF is a potent endothelial mitogen, maintains newly formed capillaries, and has important role in angiogenesis. VEGF expression is regulated by hypoxia ¹²⁹, and it exists in 5 isoforms expressed in the endometrium, but only VEGF 165 and VEGF 121 variants are shown to be modulated by sex steroids ¹³⁰. Evidence of increased VEGF expression has been documented in ovarian hyperstimulation syndrome ¹³¹. VEGF is expressed in endometrial glandular epithelium and stroma in the proliferative phase, while in the luteal phase it is expressed in the glands but not the stroma ¹³². In stroma cell culture progesterone suppresses VEGF production ¹³³. This is further explained in Chapter 9.

TGFβ

Transforming growth factor β (TGFβ) is a potent growth inhibitor, but it may stimulate the growth in malignant cell lines, including Ishikawa cells¹³⁴. It is involved in tissue differentiation and stimulates cellular production of extracellular matrix proteins¹³⁵. Its mRNA is significantly higher in the mid-late luteal and menstrual phases of the natural cycle¹³⁵. TGF β has an angiogenic effect¹³⁶, and its increased expression during the menstrual phase is probably required to stimulate capillary growth. In stromal cell culture, oestrogen and progesterone administration for 4 days is associated with an increased expression of TGFβ1¹³⁵. The mammalian TGFβ family are structurally related members designated 1, 2 and 3. TGFβ stimulates PAI-1 expression in stromal cell culture¹³⁷.

Nitric Oxide (NO)

Nitric oxide is a potent vasodilator, and may be involved in endometrial blood vessels vasodilatation, inhibition of platelet activation, and suppression of endometrial activity during pregnancy^{138, 139}. It is produced by the conversion of L-arginine to L-citrulline by the enzyme NO synthase(NOS), which exists in 3 isoforms: the endothelial cells (eNOS), neuronal constitutive (nNOS), and an inducible (iNOS), which is inducible in every cell. Oestrogen stimulates the release of NO in endothelial cell culture¹⁴⁰, and in neutrophils¹⁴¹. The expression of eNOS is low in the early to mid proliferative phase,

but is strongly expressed in the late proliferative phase and early to mid luteal phases ¹⁴².

Telfer et al. ¹⁴³, however found no difference in the expression across the natural cycle. Yoshiki et al. (2000) ¹⁴⁴ have reported on the immunolocalisation of iNOS in the endometrium being confined to the glands in the luteal phase, but become expressed in the stromal cells upon decidualization, suggesting that it may have a role in implantation.

Endometrial leukocytes

Menstruation can be viewed as a consequence of an acute inflammatory process ¹⁴⁵. Many features in the endometrium in the late luteal phase support this hypothesis, such as tissue oedema, the influx of migratory leukocytes and the presence of the decidual cells which have features of granulation tissue fibroblast ¹⁴⁶.

Endometrial leukocytes account for 10-15% of stromal cell population ¹⁴⁷. These leukocytes, some of which migrate under the effect of chemokines such as MCP-1, have different phenotypes to similar cells in the peripheral blood, and can be functionally different. Progesterone modulates leukocytic infiltration in the endometrium of the natural cycle ^{148 149} evidenced by the increase in the number of stromal leukocytes from the early luteal phase onwards ^{146 150}. Endometrial leukocytes may be involved in regulating implantation, and when this does not occur, these leukocytes may become activated to initiate endometrial disintegration and shedding ¹⁴⁷, due to the release of

cytotoxic mediators which may have a role in menstruation such H_2O_2 and $TNF\alpha$ by macrophages ¹⁵¹.

This is further explained in Chapter 8.

Tumour Necrosis factor alpha ($TNF\alpha$)

$TNF\alpha$ is a cytokine secreted by the endometrial cells. It may have a role in apoptosis, in addition to inducing vascular instability in the endometrium ending in bleeding ^{152, 153}. The mechanism by which $TNF\alpha$ acts is not very clear. Suggestion include the stimulation of epidermal growth factor ¹⁵⁴, or by stimulation of prostaglandins release ¹⁵⁵, which increase vascular permeability.

$TNF\alpha$ has been detected in the endometrium mainly in the luteal phase, in the glands and coiled arteries ¹⁵⁶. $TNF\alpha$ however, is expressed during the late proliferative phase and continues during the luteal and menstrual phases ^{157 158}, while, $TNF\alpha$ mRNA starts to peak in the late luteal phase ¹⁵⁹, hence, it is postulated to have a role in endometrial bleeding and shedding ¹⁵⁷. von Wolff et al. (1999) reported a reduction in the $TNF\alpha$ mRNA during the late luteal phase, while the $TNF\alpha$ protein was still high ¹⁵⁸. Epithelial cells are the major source of $TNF\alpha$ in-vivo and in-vitro, and cells obtained from late luteal phase endometrium secrete more $TNF\alpha$ into the medium of primary cell

culture than epithelial cells obtained from other phases of the natural cycle ¹⁵⁹.

Bcl-2 (B cell leukaemia)

Bcl-2 is a proto-oncogene whose protein product prolongs cell survival by preventing apoptosis. The Bcl2 family is a group of apoptosis regulating proteins. One of the mechanisms by which bcl-2 protects against apoptosis is the regulation of release of cytochrome-c from the mitochondria to the cytoplasm ¹⁶⁰. Accumulation of cytochrome-c promotes a sequence of events leading to the activation of caspase-9, which in turn activates pro-forms of other caspases, such as caspase-3 (CCP32), leading to cleavage of different intracellular proteins, the disruption of cellular architecture, and finally apoptosis. ^{161 162 163}.

This is further explained in Chapter 10.

Bax

Bax, a pro-apoptotic protein and a member of the bcl-2 family, with a 21% amino acid sequence homology with bcl-2 ¹⁶⁴.

The ratio of bcl2 to bax determines the sensitivity to apoptosis. When Bcl2 is in excess, bax / bcl2 heterodimers form which protect the cell from apoptosis. When bax predominates, bax homodimers are formed and the cell undergoes apoptosis ¹⁶⁵. Bax induces cytochrome c release, activation of caspase-3 and eventually apoptosis ¹⁶⁰.

Bax is expressed in the glands throughout the phases of the menstrual cycle. Endothelial cells were immunopositive throughout the cycle, while there were no staining in stromal cells or lymphocytes ¹⁶⁶.

This is further explained in Chapter 10.

Caspase-3

The caspase gene family, whose products are intracellular cysteine proteases, are involved in the process of apoptosis. When activated they cleave apoptotic substrates example and lead to cell death.

Caspase-3 (CPP32) is widely expressed in human tissue, however there are differences in the staining intensity, which seem to be tissue specific. It is expressed mainly in the cytoplasm, however in some tissues, such as colonic and prostatic epithelium, it is expressed in the nucleus ¹⁶⁷.

This is further explained in Chapter 10.

Receptor	Trimegestone	MPA	NET	Gest	LNG
rER	< 0.02	< 0.02	0.15	< 0.02	< 0.02
rPR	588	298	134	868	323
rAR	2.5	36	55	71	58

Table 1.1 The relative binding affinity of trimegestone compared to other progestogens. r = Recombinant, ER = Oestrogen receptor, PR = Progesterone receptor, AR = Androgen receptor, LNG = Levonorgestrel, NET= Norethisterone, MPA = Medroxy progesterone acetate, Gest = Gestodene.

Treatment	No. of bleeding days in the reference period (median)	Cycle length	Bleeding free interval
OCP (LNG)	13.5	27.8	24
OCP (NET)	13.5	27.8	24
E ₂ + MPA	15.6	29	24
E ₂ + NET	15.6	29	24
IVR	17.5	25.8	20.6
Depo-MPA	11	35.9	27.4
Control	19	28	22

Table 1.2. Bleeding parameters in women on different contraceptive methods. OCP = Oral contraceptive pill, EE = Ethinyloestradiol, LNG = Levonorgestrel, NET= Norethisterone, MPA = Medroxy progesterone acetate, IVR = intra-vaginal ring.

Chapter Two

Aims of the research programme

The analysis of menstrual diaries of women treated with sequential combined trimegestone-oestradiol HRT regimen in a dose ranging study for 6 months, demonstrated a strong dose-dependent effect of trimegestone on the bleeding pattern experienced by these women. Endometrial histology at the end of the study, however, showed secretory changes in 96% of biopsies collected. The issue of compliance with study medications was addressed both in terms of drug accountability and serum trimegestone levels at each visit. Further documentation of trimegestone dose dependence of bleeding patterns was evident in a 6-month extension study of the HRT regimen using a single dose of the progestogen. These findings led me to hypothesise that features, other than orthodox histological assessments, might operate within the endometrium characteristic of the effect of trimegestone, and/or its dose, which are responsible for this highly variable bleeding pattern recorded by the participating women in the study.

The influence of endometrial structural abnormalities on the pattern of bleeding was evaluated in the subset of trial population who underwent hysteroscopic assessment at baseline and at the end of the dose-ranging study. Submucous fibroids, influenced the incidence of inter-menstrual bleeding, however, the dose of trimegestone remained the strong determinant of the pattern of the bleeding.

The plan of investigations was structured along the lines of evaluation of the extent of endometrial evolution in biopsies obtained on a fixed day of the trimegestone administration (day 10). The data were then compared to the findings in a set of highly documented endometrial samples obtained during the natural cycle. The aims were to document these changes in:

- a. Histomorphometric analysis of endometrial glands and stroma.
- b. Endometrial leukocytic infiltration and cellular subtypes.
- c. The vascularity of the endometrium as assessed by CD-34 expression.
- d. The prevalence of apoptosis-promoting and inhibitory markers.
- e. The pattern of expression of metalloproteinases.

Since a dose dependent effect of trimegestone on the endometrium was not documented in the above studies, together with the identification of general patterns of similarity to the luteal phase of the natural cycle, the question arose as to how identical trimegestone is to progesterone, in its effect on the endometrium? In an attempt to answer this question, I have optimised and established a primary stromal cell culture system, and subjected them to series of sex steroid treatments. The target genes reported in this thesis are those of MMP-1, MMP-3, and of GAPDH as a housekeeping gene.

Chapter Three

Methods

The dose ranging study of trimegestone

This is a multicentre, prospective, parallel group, double-blind, randomised, dose ranging study to optimise the dose of sequentially added trimegestone to oestradiol with regard to uterine bleeding and endometrial safety. The protocol was approved by every local ethics committee and all patients signed informed consent.

Women included in this study were healthy, aged 45-65 years (mean \pm SD, 52.5 ± 5.1), with an intact uterus whose menopausal status should have fulfilled one of the following criteria: 1. at least six months had elapsed since the last menstrual period, with FSH and oestradiol levels in the postmenopausal range. 2. had used HRT continuously for more than two years or 3. had been on HRT for at least one year with pre-treatment FSH and oestradiol levels in the postmenopausal range.

None had received any form of sex steroid treatment for 6 weeks before the commencement of study medications. Those who had ever used oestradiol implants were excluded. Tests for liver and renal function were performed and those women with abnormalities were excluded.

All women over the age of 50 had a normal mammogram within three years, and normal cervical smears within the previous 6 months. General, breast, and pelvic examinations were conducted to confirm normality. Pelvic sonography was performed to measure the size of the uterus, endometrial thickness and to assess the adnexa. All endometrial biopsies were evaluated by two independent pathologists, blinded to the dose of trimegestone. Endometrial biopsy using the Vabra curette was performed at the screening visit. Women were excluded if the histology showed any evidence of hyperplasia or cancer.

During the study, additional sex steroid treatments were not allowed and women who used treatments known to interfere with steroid metabolism were withdrawn. At the final visit, general, breast, and pelvic examination were repeated, pelvic sonography and endometrial biopsy were performed on day 24 of the last treatment cycle. The endometrial biopsies at the end of the study were evaluated by two independent pathologists. Women were randomised to receive oral micronised oestradiol 2mg/day (RU 3499) for 28 days and one of four doses of oral trimegestone (0.05, 0.1, 0.25, or 0.5 mg; Hoechst Marion Roussel, Romianville, France) from days 15-28.

The study population addressed in chapters 6 to 11 was a subset of dose-ranging study of trimegestone described above. It included women who attended the Menopause Research Unit (MRU), Leicester, and were subjected to the same inclusion and exclusion criteria described above. There were no statistically significant differences between this subgroup of 176 women and the total study population of 256 women in terms of age, race, height, weight, body mass index, smoking habits, and time since menopause.

Further, this group of women underwent hysteroscopy under local anaesthesia with CO₂ gas distension at baseline and at the end of the study. This diagnostic addition to the protocol was approved by the Leicestershire ethics committee and the procedure was included in the information sheet and in the signed informed consent form.

The Control Endometrial Samples

The control samples were deep endometrial biopsies (which included functionalis and basalis layers), obtained from healthy, regularly menstruating women aged 27-50 (mean \pm SD, 38 ± 6.1), undergoing laparoscopic sterilisation using sharp curette, or from hysterectomy specimens. The clinical indications for the hysterectomies were: cervical intraepithelial neoplasia, premenstrual tension syndrome and benign ovarian cysts. None of these women had received any hormonal treatments for 2 months prior to the procurement of the specimens. All women were given urinary LH surge detection kit tests (First Response, Carter Wallace limited, Folkstone, UK), which were used during the month preceding the endometrial biopsy or hysterectomy.

The technique I used to obtain endometrial samples from hysterectomy specimens is as follows: the uterus is opened in the coronal section and the posterior wall of the uterine cavity is sliced vertically from fundus to isthmus, each slice being 1 cm wide and are numbered alphabetically A - E from right to left. My study material are from slice B (Figure 3. 1). I have chosen to take the endometrial samples from that specified area of the uterus to maximise consistency, since the fundal region is recognised to be the most hormone responsive area of the endometrium, and decided to specify area B as this avoids the lateral edge of the fundal endometrium which may sometimes undergo tubal epithelial metaplasia.

The control specimens were fixed immediately in 10% formal-saline, embedded in paraffin wax, and 5 µm sections were stained with Hematoxyline and Eosin (H&E) for histological assessment of the phase of endometrial development.

These biopsies were dated both by LH surge and the date of the last menstrual period, and were examined by 2 independent pathologists who were blinded to the LH surge and menstrual dates. Where all agreed, the specimen was included as a control sample.

Tissue preparation

Formalin fixation

Endometrial tissue were fixed in 10% buffered neutral formalin solution, then tissue were processed automatically through dehydration with grades of ethyl alcohol, several washes in chloroform, then washing in xylol. Followed by embedding in paraffin wax. Sections of 5µm thick were cut using Leica RM2035 microtome and suspended on silane coated slides (3-Aminopropyl-Triethoxy-Silane, Sigma, Dorset, UK). Sections were dried overnight in a 37⁰C heated oven.

Hematoxylin and Eosin

Sections were dewaxed in xylene (for 5 minutes), rehydrated in 99%, 90%, 70% ethyl alcohol (for 5 minutes), then washed in distilled water for 5 minutes, followed by staining with Harris hematoxylin (Sigma, Dorset, UK), for 5 minutes , then washed in running tap water.

Sections were stained in Eosin (TAAB laboratories, Berkshire, UK), then washed in running water before dehydration by washing in alcohol in reverse order to the rehydration steps. Cover slip No. '0' glass was used to cover the sections using DPX mountant (contains distrene 80, dibutyl phthalate and xylene).

Immunohistochemistry (IHC)

Immunohistochemistry techniques used in chapters 7 to 11 of this thesis were performed on the biopsies which were obtained from the subgroup of women who participated in the trimegestone dose ranging study at the Menopause Research Unit, Leicester (page 52).

Immunohistochemistry is a method for the detection of an antigen with a specific antibody (Table 3. 1).

Standard single labelling for Ki-67

1. Sections were de-waxed as follows;

Xylene de-wax 3 minutes

Xylene for 3 minutes

Xylene for 3 minutes

99% alcohol for 3 minutes

99% alcohol for 3 minutes

95% alcohol for 3 minutes

2. Sections were washed in distilled water (dH₂O) for 3 minutes to re-hydrate.

3. The slides were immersed in 10mM citrate buffer at pH 6.0, and heated in the microwave oven at 750 Watts for 30 minutes, followed by cooling and washing in tap water.
4. Endogenous peroxidase was blocked by incubating the sections in freshly prepared, cold, H₂O₂ 6% v/v for 10 minutes, followed by washing in running tap water for 5 minutes. The slides were washed in phosphate buffer saline (PBS) for 5 minutes.
7. Slides were wiped around the tissue to absorb excess buffer and covered with 100µl/slide of Normal Rabbit Serum (NRS, Dako, Glostrup, Denmark), diluted 1:10 in PBS, and left at room temperature (RT) in a humidity chamber for 30 minutes.
8. Excess NRS was wiped off and the primary antibody (MiB-1) at 1:150 dilution in blocking solution was added at 100µl/slide, and kept in a humidity chamber overnight at RT.
9. Slides were washed in PBS for 20 minutes, and then incubated with 100µl of the secondary antibody, biotin-linked rabbit anti mouse IgG (DAKO, Glostrup, Denmark) at 1:400 dilution in PBS. Incubation was in a humidity chamber for 30 minutes at RT.
Vectastain ABC peroxidase solution was mixed (Vector laboratories, Peterborough, UK) at least 30 minutes before its use, as follows; to 5mls of PBS, 2 drops of solution A are added and mixed, then 2 drops of solution B and mixed. Slides were washed in PBS for 20 minutes.
10. Vectastain ABC peroxidase was added at 100µl and incubated at RT for 30 minutes and washed in PBS 30 minutes.
11. Sections were incubated with peroxidase substrate Diaminobenzidine (DAB, Vector laboratories), for 5 minutes.

DAB was prepared according to manufacturer recommendations.

Sections were washed in running tap water for 5 minutes.

12. Sections were incubated in 0.4% copper sulphate for 5 minutes, then washed in running tap water for 5 minutes.

13. Counterstaining sometimes was used with Mayer's haematoxylin for 20 seconds, followed by washing in tap water.

14. Sections were dehydrated and cleared by washing in 95% alcohol for 3 minutes, 99% alcohol for 3 minutes, 99% alcohol for 3 minutes, Xylene for 3 minutes, and lastly Xylene for 6 minutes.

15. Slides were mounted in XAM (BDH Merck Ltd, Lutterworth, UK), and covered with the appropriate cover slip.

Immunohistochemistry for CD56: was similar as above, but the primary antibody was diluted 1: 25 and incubated at RT.

Immunohistochemistry for CD34: was similar as above, but the primary antibody was diluted 1: 100 and incubated at RT.

Immunohistochemistry for Bcl-2: was similar as above, but the primary antibody was diluted 1: 25 and incubated overnight at 4°C.

Immunohistochemistry for BAX: was as above but differ as following;

- Heating in the microwave oven was for 12 minutes only
- Blocking was with Normal Goat Serum (DAKO, Glostrup, Denmark) at 1: 20.
- Primary antibody was diluted 1: 200 and incubated overnight at 4°C.

- Secondary antibody was Goat anti-rabbit IgG.

Immunohistochemistry for Caspase-3: was as above but;

- Washing in H_2O_2 6% v/v was omitted.
- Primary antibody was diluted 1: 100 and incubated overnight at 4°C.
- Vectastain ABC-Alkaline phosphatase (ABC-AP, Vector laboratories, CA, USA) was used for 30 minutes.
- The substrate (BCIP/NBT, Vector lab, Peterborough, UK) was prepared according to manufacturer recommendations and added. The slides were kept in the dark for 30 minutes.

Immunohistochemistry for MMPs , -1, -3 and -9

As above but;

- Blocking was with NGS 1: 20 dilution in TBS for 10 minutes.
- The primary antibody was incubated overnight at 4° C.

MMP-1: 2µg/ml

MMP-3: 3µg/ml

MMP-9: 3µg/ml

- Secondary antibody was goat anti-mouse IgG at 1: 400 dilution.

Immunohistochemistry for CD45 will be explained as it is different from above:

1. De-waxing and rehydration were as above.
2. Slides were washed in dH₂O for 3 minutes to re-hydrate
3. Sections were washed in 0.4% pepsin HCl & incubated at 37°C for 20 minutes, followed by washing in running tap water for 5 minutes to remove the pepsin.
4. Endogenous peroxidase was blocked by incubation in freshly prepared H₂O₂ 6% v/v for 10 minutes, followed by washing in running tap water for 5 minutes, and the slides were washed in PBS / 0.05% Tween 20 for 5 minutes.
5. Slides were wiped around the tissue and covered with 100µl/slide of NRS (Dako, Glostrup, Denmark) diluted 1:10 in PBS, and left at RT in humidity chamber for 20 minutes.
6. Avidin D solution (100µl/slide, Vector laboratories, Peterborough, UK) was added and incubated in a humidity chamber at RT for 15 minutes. Avidin D solution is prepared by adding 4 drops of Avidin D solution to 1.0 ml of PBS buffer, followed by washing in PBS / 0.05% Tween 20 for 5 minutes.
7. Excess liquid was wiped off and Biotin solution (Vector laboratories, Peterborough, UK) was added at 100µl/slide (Biotin solution was prepared by addition of 4 drops of Biotin solution to 1.0 ml of PBS buffer). Slides were incubated in a humidity chamber at RT for 15 minutes. Sections were washed in PBS / 0.05% Tween 20 for 5 minutes
8. Excess liquid was wiped off and 100µl of anti-CD45 antibody at 1:150 dilution in NRS/PBS (1:10) was added/slide and incubated in a humidity chamber overnight at 4°C.

9. Steps were continued as for immunohistochemistry for ki-67 from step 9 onwards.

Immunohistochemistry for CD3: was as for CD45 but:

- Pepsin digestion was for 40 minutes.
- Normal Swine Serum was used instead of NRS.
- Anti-CD3 antibody in dilution of 1: 50 and incubated at 4°C.

Double labelling for ki-67 and CD45

1. Sections were de-waxed and re-hydrated to water in the fume hood as for single labelling.
2. Sections were pre-treated in 0.4% pepsin HCL solution for 20 minutes at 37°C, then washed in running tap water for 5 minutes, followed by washing in PBS for 5 minutes.
3. Slides were wiped around the tissue and covered with 100µl/slide of NRS (Dako, Glostrup, Denmark) diluted 1:10 in PBS, and left at RT in a humidity chamber for 20 minutes.
4. Slides were wiped around the tissue and 100µl of primary antibody (anti-CD45, LCA) was added at dilution of 1: 150 and incubated overnight at 4°C.
5. Slides then were washed in PBS for 20 minutes.
6. Excess fluid was wiped off and 100µl secondary antibody rabbit anti mouse alkaline phosphatase-linked IgG was added at a dilution of 1:100

in TBS and incubated in a humid chamber for 30 minutes. Slides were washed in PBS for 20 minutes.

7. Alkaline phosphatase substrate, Fast red TR/Naphthol AS-MX (Sigma, Dorset, UK) was added for approximately 20 minutes. Sections were washed in running tap water for 5 minutes.

8. Slides were immersed in 6M Citrate Buffer and heated in the microwave oven at 750watts for 30 minutes, followed by cooling and rinsing in cold water. Sections were washed in PBS for 5 minutes.

9. Slides were wiped around the tissue and covered with 100µl/slide of NRS (Dako, Glostrup, Denmark) diluted 1:10 in PBS, and left at RT in a humidity chamber for 15 minutes.

10. Primary antibody, MIB-1 was added at a dilution of 1:150 in (NRS in PBS) overnight at RT. Slides were washed in PBS for 20 minutes.

11. Slides were wiped around the tissue and covered with 100µl secondary antibody, rabbit anti-mouse IgG diluted at 1:400 in PBS and incubated in a humid chamber for 30 minutes. Slides were washed in TBS for 20 minutes.

12. 100µl of Vectastain ABC peroxidase solution/slide was added and left for 30 minutes followed by washing in PBS for 30 minutes.

13. Blue/grey peroxidase substrate (Vector, Laboratories, CA, USA) was added to the sections after preparation according to manufacturer specification, and slides were incubated for approximately 5 minutes, or until colour fully develops.

14. Sections were washed in running tap water for 5 minutes, then put in distilled water and mount using Aquamount (BDH Merck Ltd, Lutterworth, UK).

Quality control in immunohistochemistry

Positive and negative controls were used at tissue and antibody levels. For example, tonsils were used as positive controls for CD45 and ki-67 antigens, and for both these antigens, sections of ligamentous tissue acted as negative control. At the level of primary antibody, replacement with allogenic non-specific IgG (Sigma, Dorset, UK) when monoclonal antibody were used, as negative control, or allogenic non-immune serum (Dako, Glostrup, Denmark) in the case of polyclonal antibodies. To control for the secondary antibody was to omit the primary antibody.

Numbers of fields assessed

I assessed the number of fields per slide required to be examined when detecting each antigen in order to account for the variability within specimens to detect a statistically significant difference of $\alpha = 0.05$ and $\beta = 0.8$, given that I have examined all specimens available to me for each stain, to reduce variability between the slides and that is necessary for a statistical power of $\alpha = 0.05$. I used the following equation:

$$\frac{1}{N_s} \times (V_s + \frac{V_f}{N_f})$$

N_s = No. of slides

V_s = variance between slides

V_f = variance between fields

N_f = No. of fields

Evaluation of the tissue sections

Images were captured using Axioplan microscope (Carl Zeiss, Herts, UK), and a colour video camera (Sony CCD/RGB). The areas measured or cells counted were evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, UK).

Assessment of reproducibility of the histomorphometric measurements

The reproducibility of the endometrial histomorphometric measurements (Chapter 7) was evaluated. Ten slides were chosen at random to be examined by a second observer. The images of the randomly selected fields from each slide ¹⁶⁸ were stored on the computer. The histological parameters assessed were; total glandular area, the height of glandular epithelial (columnar or cuboidal), percent of glands containing luminal secretions or subnuclear vacuoles and the regularity of the glandular epithelial surface (smoothness).

To plot the 2 sets of results on the X and Y axes, and to draw the line of equality will result in the data being scattered along the line, and may even give a false impression of correlation. This is particularly true since this approach to the data does not take into account the observer's bias in deducing these true measurements.

Each observer produced a mean value of measurements for each slide. I plotted the differences between the means against the average of these 2 mean values, which removed the individual observer's bias ¹⁶⁹.

Results (Figures 3.2a-f)

Assessment of the differences between 2 observers (MW and FAL) was based on the measurement of the difference between the means divided by the standard error of the difference between the means, which determined the *t* value.

For n-1 degrees of freedom, in this case =9, the *t* distribution corresponding to P < 0.05 is 2.26. Where the *t* value is < 2, the difference between the observers fails to reach statistical significance.

<u>Parameters</u>	<u>No. of agreed slides (out of 10)</u>
Total glandular area	9
Height of the glandular epithelial (columnar)	6
Height of the glandular epithelial (cuboidal)	5
% of glands with vacuolation	7
% of glands with secretions	10
% of glands with smooth lining	9

Summary of the Statistics

The methods used for statistical analysis are detailed in the relevant chapters. In summary; the unpaired t-test and ANOVA was used for statistical analysis of continuous variables, which fulfilled the definitions of normal distribution. The non-parametric, Kruskal-Wallis and Mann-whitney tests were used when the data were not normally distributed.

Linear discriminant analysis (LDA) was utilised to discriminate between doses of trimegestone and the phases of the natural cycle based on endometrial histological and immunohistochemical measurements as described in the relevant chapters. Direct comparisons of individual parameters involved in a complex tissue dynamics such as those of endometrial proliferation and differentiation will certainly miss the overall picture. Intra-specimen variations (different fields may show different maturational steps) and the variable tissue responses between women complicate this issue further.

LDA was adopted because it can cope with intra- and inter-subject variability and can handle such scatter for population. The actual details of derivation of weighted variables and their scatter are depicted in Appendix B.

The data concerning the mRNA gene expression in stromal cell cultures were analysed using mixed model for the log of the ratios, with Wald tests used to compare treatment effects.

Stromal cell culture

Stromal cells were isolated from luteal phase endometrium obtained from hysterectomy specimens^{170 171}. Endometrial samples were washed with HBSS (GibcoBRL, Paisley, Scotland) and minced finely into small pieces, then incubated in 20ml of 0.25% bacterial collagenase type I solutions (Gibco BRL, Paisley, Scotland), for 2 hours at 37°C. Cells were washed x 6 in growth medium; phenol free DMEM/F-12 medium (Gibco BRL, Paisley, Scotland) supplemented with charcoal stripped 10% fetal calf serum (Hyclone, Logan, UT, USA), antibiotics /antimycotic (Penicillin 10.000 units, 10 mg streptomycin, 25µg Amphotericin B/ml, Sigma, Irvine, UK) and 15 ml sodium bicarbonate (Sigma, St.Louis, USA). Cells were allowed to settle down and the top 16 mls containing mainly stromal cells was removed, and centrifuged at 1500rpm for 5 minutes. The cellular pellet was resuspended in growth medium and seeded into a 25 mm² flask (NUNC, IL, USA) and incubated at 37°C in 5% CO₂ in humidified air until confluence, usually 5 to 6 days later. The cells were removed with trypsin-EDTA (Gibco BRL, Paisley, Scotland), washed twice with growth medium, resuspended and plated into 6-well plates at cell density of 8-10 x 10⁴ cells/ml. Cells were also seeded on 3 glass slides (C.A.Hendley Ltd. Essex. UK) for immunocytochemical staining for vimentin. The cells in the 6-well plates and on the glass slide were adherent after 24 hours, when they were washed and the medium was changed

Drugs used in the experiments (Figure 3.3 a & b)

All steroids were dissolved in 99% ethanol first then diluted in DMEM/F-12 medium . Oestradiol 17 β (10^{-6} M) was added to cell culture wells for 4 days. Controls included equimolar concentration of Oestradiol 17 α (10^{-6} M, isomeric control), or equal volumes of 99% ethanol. All material were obtained from Sigma, St.Louis, USA. The culture medium was changed every second day with the same additives.

The second phase of the experimental design involved the addition of 10^{-6} M progesterone (Sigma, St.Louis, USA), 10^{-6} M trimegestone (Hoechst Marion Roussel, Romianville, France) and or 10^{-6} M RU486 (Hoechst Marion Roussel, Romianville, France) from day 5 to 10 or day 5 to 8 in different combinations.

Immunocytochemistry for vimentin

Stromal cells were stained with anti-vimentin antibody to confirm their stromal origin. When the cells covered 60-70% of the dimple of the slide, they were immersed in 99% ethanol for 10 minutes and then air dried. Positive staining to vimentin indicates that the cells had the characteristics of stromal cell ^{170 172 173}.

Technique

1. Endogenous peroxidase activity was blocked by immersing the slides in cold H₂O₂ 6% v/v, 10 minutes, followed by washing in tap water for 5 minutes.

2. 100 μ l of normal goat serum was added at 1: 20 dilution in TBS for 10 minutes at RT.
3. Slides were wiped around the area of fixed cells to mop out excess buffer. Anti-vimentin mouse monoclonal antibody, 1:100 (Novocastra, Vector Laboratories Ltd, Peterborough, UK) was added to these slides and were incubated in a humidity chamber at 4°C overnight, followed by washing in PBS for 20 minutes. To one control sample non specific mouse IgG 5 μ g/ml was added and to the other, the primary antibody was omitted.
4. Slides were wiped around the area of fixed cells and biotinylated goat anti-mouse secondary antibody (DAKO, Glostrup, Denmark) diluted 1:400 in PBS was added for 30 minutes at RT, then washed in PBS for 20 minutes.
5. ABC vectastain was added at 100 μ l / slide for 30 minutes, followed by washing in PBS for 20 minutes.
6. Sections were incubated with peroxidase substrate DAB (Vector Laboratories Ltd, Peterborough, UK), for 5 minutes. Sections were then washed in tap water for 5 minutes.
7. Counter-staining was with Mayer's hematoxylin (Sigma, Dorset, UK) for 30 seconds, then sections were washed in tap water.
8. Sections were dehydrated and cleared.
9. Slides were mounted in Xam, and covered with cover slips.

Extraction of mRNA using dynabeads

Stromal cells were cultured in triplicate wells, and the sample from each well was divided into 3: 2 samples were run as RT +ve and one sample as RT-ve

1. The mRNA was collected from each well by the addition of 500µl lysis binding buffer (LBB).
2. To 100µl of lysate, proteinase K in DEPC was added to a final concentration of 50µg/ml (5µl of a 1 mg/ml stock proteinase K in DEPC solution). Tubes were incubated at 37°C for 1 hour.
3. The DNA was sheared by passing preparation through a 21 G, followed by a 25G sterile needle in a 1 ml syringe, 3 to 5 times each.
4. Samples were microfuged at 1500 rpm for 30 seconds to reduce frothing.
5. 30µl of Dynabeads Oligo (dT), designed to isolate poly A⁺ RNA from total RNA (Dynal company, UK) per sample were placed into the Dynal Magnetic Particle Concentrator (Dyna MPC, Dynal company, UK) to pellet the beads, and the supernatant was discarded and 30µl of LBB was added.
6. Step 5 was repeated, then 30 µl of the Dynabeads were added to each sheared lysate.
7. Samples were left for 5 minutes at RT to allow mRNA to bind to the beads.
8. Samples were allowed to pellet in the MPC, and the supernatant was discarded. The pellets were washed twice in 2 volumes (60µl) of wash buffer with LiDS, followed by further 2 washes, 60µl each with buffer without LiDS.
9. Lastly samples were resuspended in 30µl DEPC water, and stored at 4°C until the Reverse Transcriptase (RT) reaction, which was performed on the same day as the mRNA extraction.

Reverse Transcriptase reaction using mRNA

RT reactions were carried out on the mRNA extracted onto Dynabeads, to produce complementary DNA, which can then be amplified by PCR.

Master mix solutions: All products from (Hybaid-AGS, Ashford, Middlesex, UK). The primer was the oligodeoxythymidine attached to the beads.

	+RT reaction	-RT reaction
mRNA / beads preparation	10 μ l	10 μ l
DMSO	1 μ l	1 μ l
5 x AMV- buffer	2.5 μ l	2.5 μ l
10 mM dNTPs / DEPC	0.5 μ l	0.5 μ l
RNasin (25U)	0.25 μ l	0.25 μ l
DEPC water	10.25 μ l	10.75 μ l
AMV-RT (5U)	0.5 μ l	-----

15 μ l of the master mix were added to 10 μ l of the sample, and incubated at 42°C for 1 hour, using a Hybaid PCR machine on Manual setting, then stored at 4°C.

Polymerase chain reaction (PCR)

The thermal cycler protocol, regarding annealing temperatures and number of cycles was optimised for each primer set (Figure 3.4a, b & c)

1. The master mix was prepared for the number of samples + 1 reactions.

Distilled water	42 μ l
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Alex Jeffrey buffer	5µl
Primers containing solutions	2µl

The primer sequences for MMP-1, MMP-3 and GAPDH are detailed in Table 3.2. (10pmol of primer was used per sample)

2. 49µl of the master mix was added to 1µl of the sample in an eppendorf tube.
3. 30 µl of mineral oil was added to each tube and the tubes were sealed.
4. The tubes were placed in the thermal cycler (Hybaid-AGS, Ashford, Middlesex, UK).
5. The PCR programme for MMP-3 was as follows:

Stage 1	step 1	94°C	2min.
Stage 2	step1	94°C	30sec.
	step2	56°C	10min. (1 cycle).

2µl (1unit) of Taq polymerase (Premega, Southhampton, UK) was added.

The reaction was completed as follows:

Stage 1	step1	94°C	30sec (denaturing step)
	step2	56°C	30sec (annealing temperature)
	step3	70°C	45 (polymerase extension)
			for 9 cycles
Stage 2	step1	94°C	30sec

	step2	56°C	30sec
	step3	70°C	45 sec
			for 13 cycle
Stage 3	step1	70°C	10min.

Amplification of MMP-1 and GAPDH followed the same steps mentioned above. However, the annealing temperatures were 62°C and 60°C and number of cycles were 32 and 28 for MMP-1 and GAPDH primers, respectively for each primer.

Gel electrophoresis

Amplified DNA bands were separated by agarose gel (3%) electrophoresis. Agarose MP, 3g (Boehringer Mannheim, Germany) were added to 100 ml of 1X TAE containing Ethidium bromide (ICN Biochemicals, Ohio, USA, 0.5µg/ml) 5µl per 100ml of 1mg/ml stock solution.

2. The mixture was weighed and brought to boiling point by heating in a microwave oven.
3. The mixture was weighed again and the difference is replaced with the addition of TAE to bring the mixture to the original weight.

6µl of the PCR sample were mixed with 2 µl gel loading buffer. The mixture was loaded onto 3% agarose gel, which was submerged in 1250

ml electrophoresis buffer containing Ethidium bromide. The samples were run for 90 minutes using power pack at 100 Volts.

Evaluation of the resolved bands

The images of the resulting bands on the agarose gel were captured by videocapture UVP camera (UVP, Cambridge, UK) using UVP image store 5000 (UVP, Cambridge, UK) and quantified by using Scion analysis scanning software (NIH).

Measurement of MMP-1 and -3 in tissue culture supernatant

MMP-1 and MMP-3 were measured in the culture medium by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (Amersham Pharmacia Biotech UK ltd, Buckinghamshire, UK). The assay was performed in duplicates from each well, and sets of triplicate wells each represented the control and individual treatment mode of the experiment series.

Antibody	Concentration	Company	incubation cond.
Ki-67	1:150, mouse, monoclonal	Pharmingen, St. Diego, California, USA	RT
CD45	1:150, mouse, monoclonal	DAKO, Glostrup , Denmark	4 ⁰ C
CD3	1:150, rabbit polyclonal	DAKO, Glostrup, Denmark	4 ⁰ C
CD56	1:25, mouse, monoclonal	Novocastra, Newcastle upon Tyne, UK	RT
CD34	1: 100 (2µg/ml) mouse monoclonal	Santa Cruz Biotechnology, California, USA	RT
Bcl-2	1: 25, mouse monoclonal	DAKO, Bucks, UK	4 ⁰ C
Bax	1: 200, rabbit polyclonal	Santa cruz, California, USA	4 ⁰ C
MMP-1, -3, -9	2-3 µg/ml, mouse monoclonal	Chemicon international Ltd, Harrow, UK	4 ⁰ C
Caspase-3	1 : 100, mouse monoclonal	Transduction laboratories, Exeter, UK	4 ⁰ C

Table 3. 1. The antibodies used in immunohistochemistry. RT = room temperature.

Primer name

Sequence

GAPDH forward AGAACATCATCCCTGCCTC

GAPDH reverse GCCAAATTCGTTGTCATACC

MMP1 forward 3' ATG AGC CGC AAC ACG ATG TAAG

MMP1 reverse 5' AAG GTG ATG AAG CAG CCC AGA T

MMP3 forward 3' CAG TGT TGG CTG AGT GAA AG AG

MMP3 reverse 5' TCT GAA AGT CTG GGA AGA GGTG

Table 3. 2. The sequence of the primers used in PCR reactions.

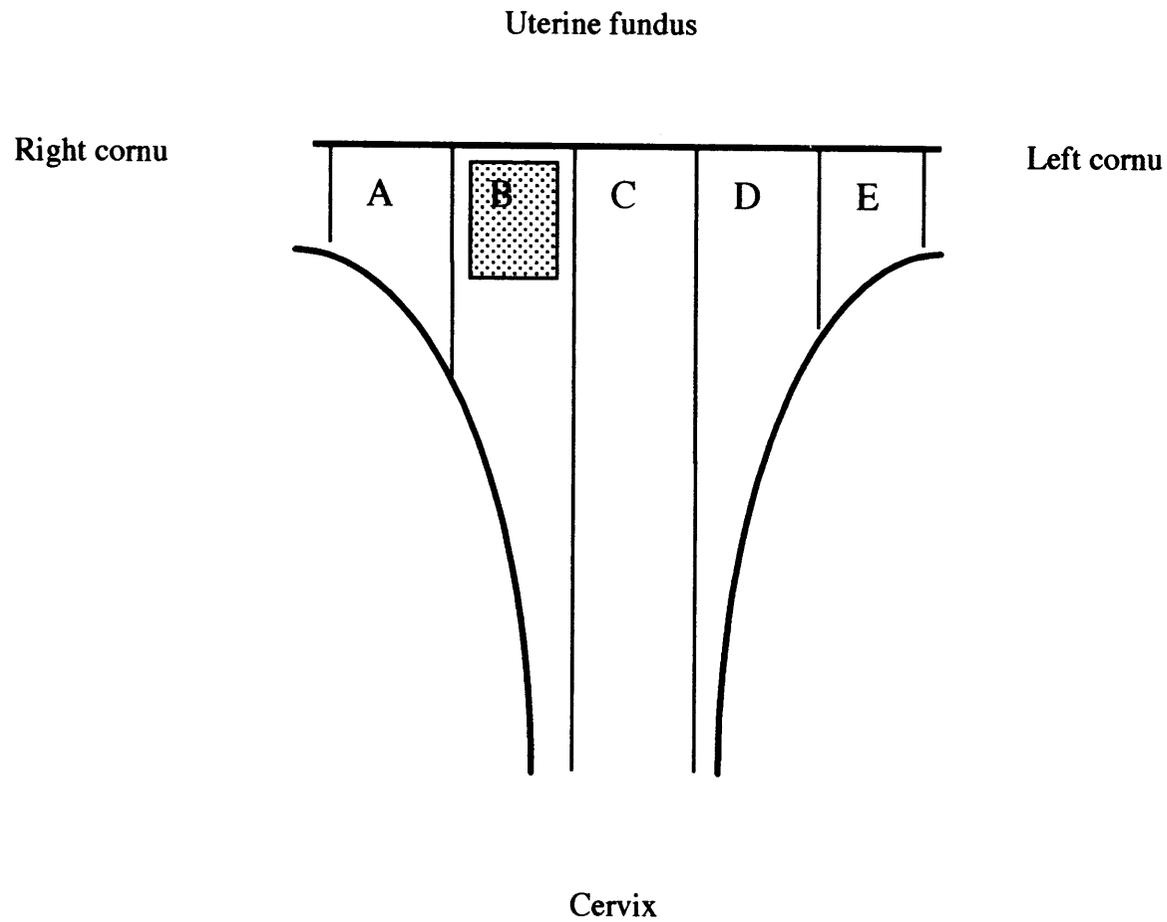


Figure 3.1: The source of the control sample (area B), in hysterectomy specimen.

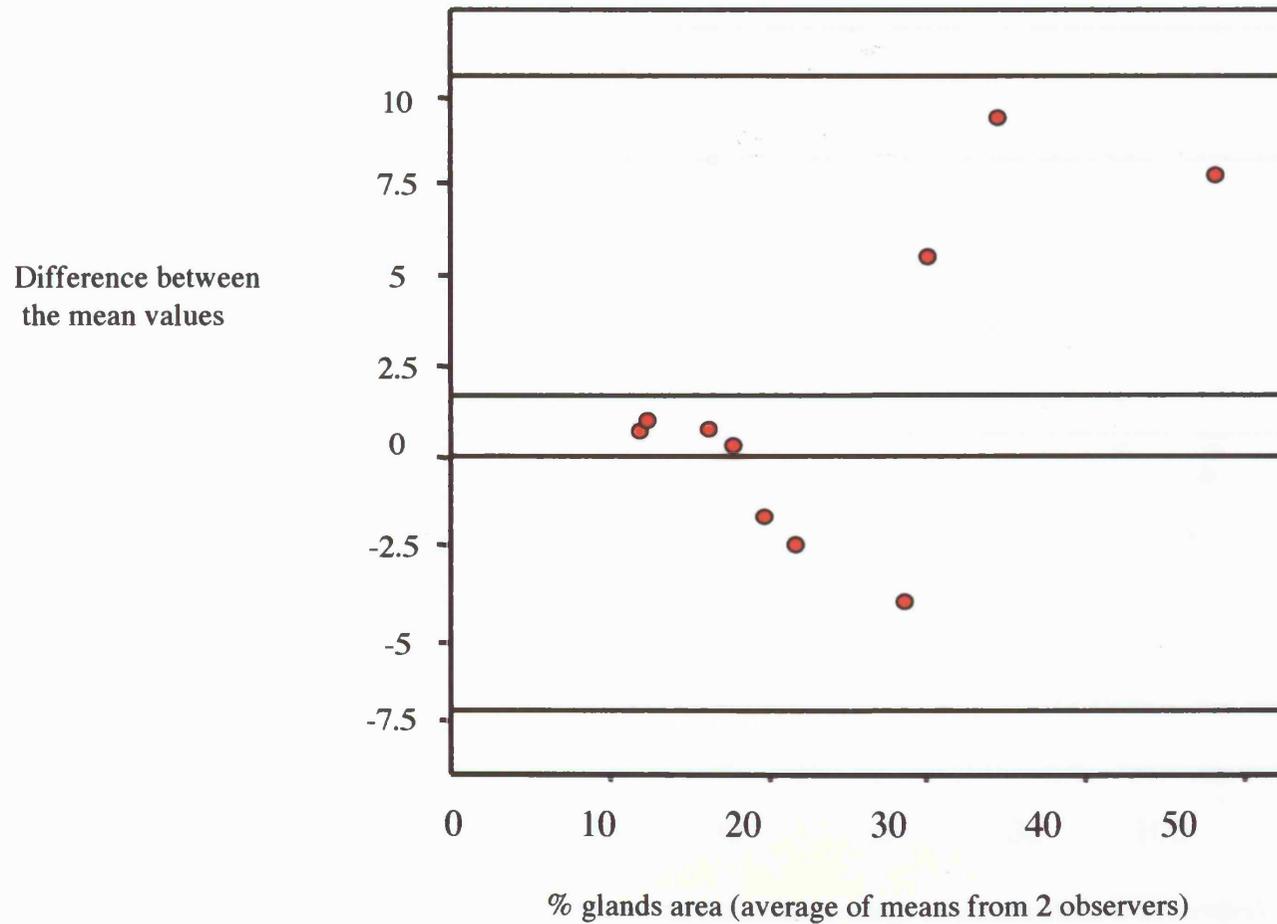


Figure 3.2a: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

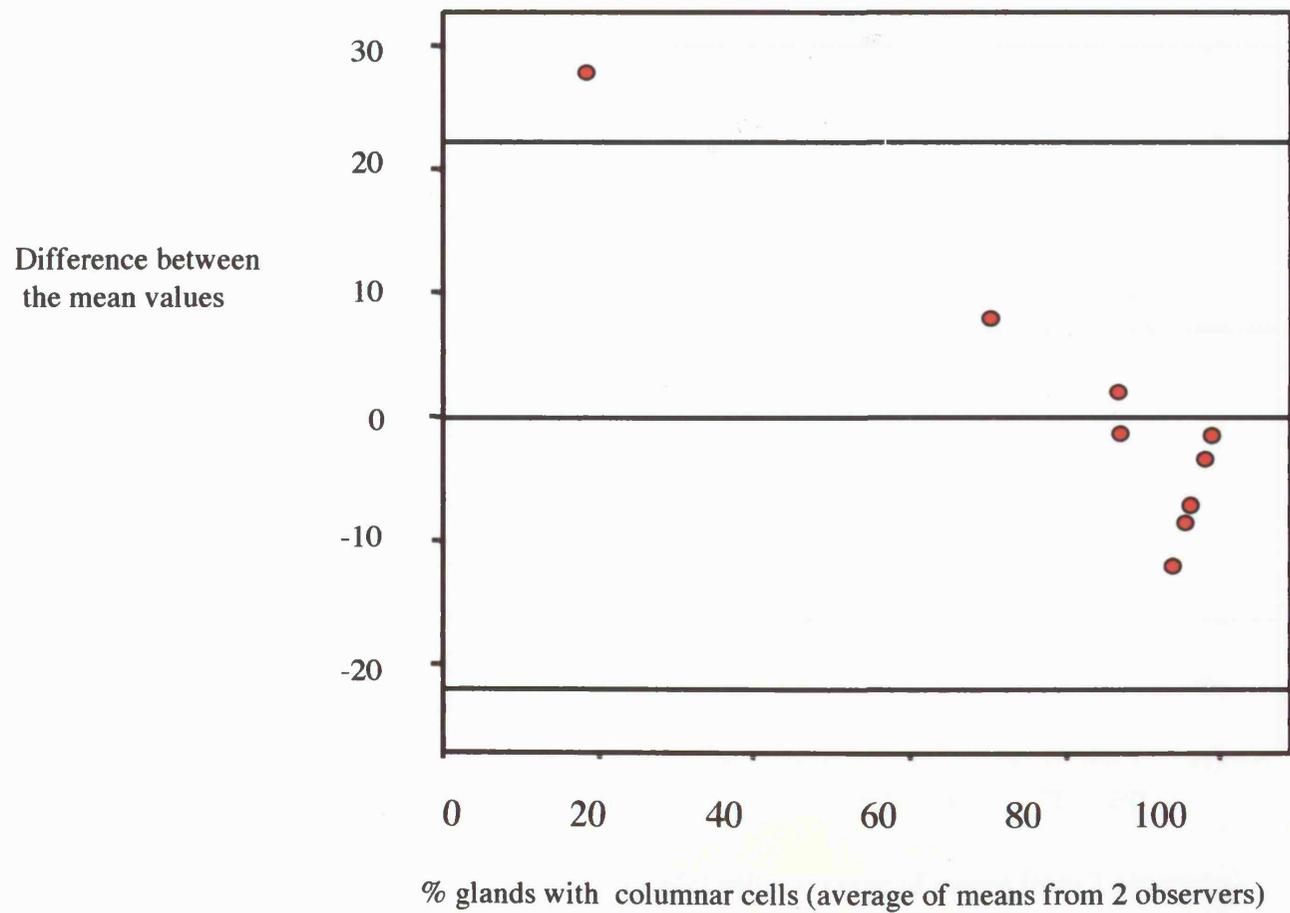


Figure 3.2b: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

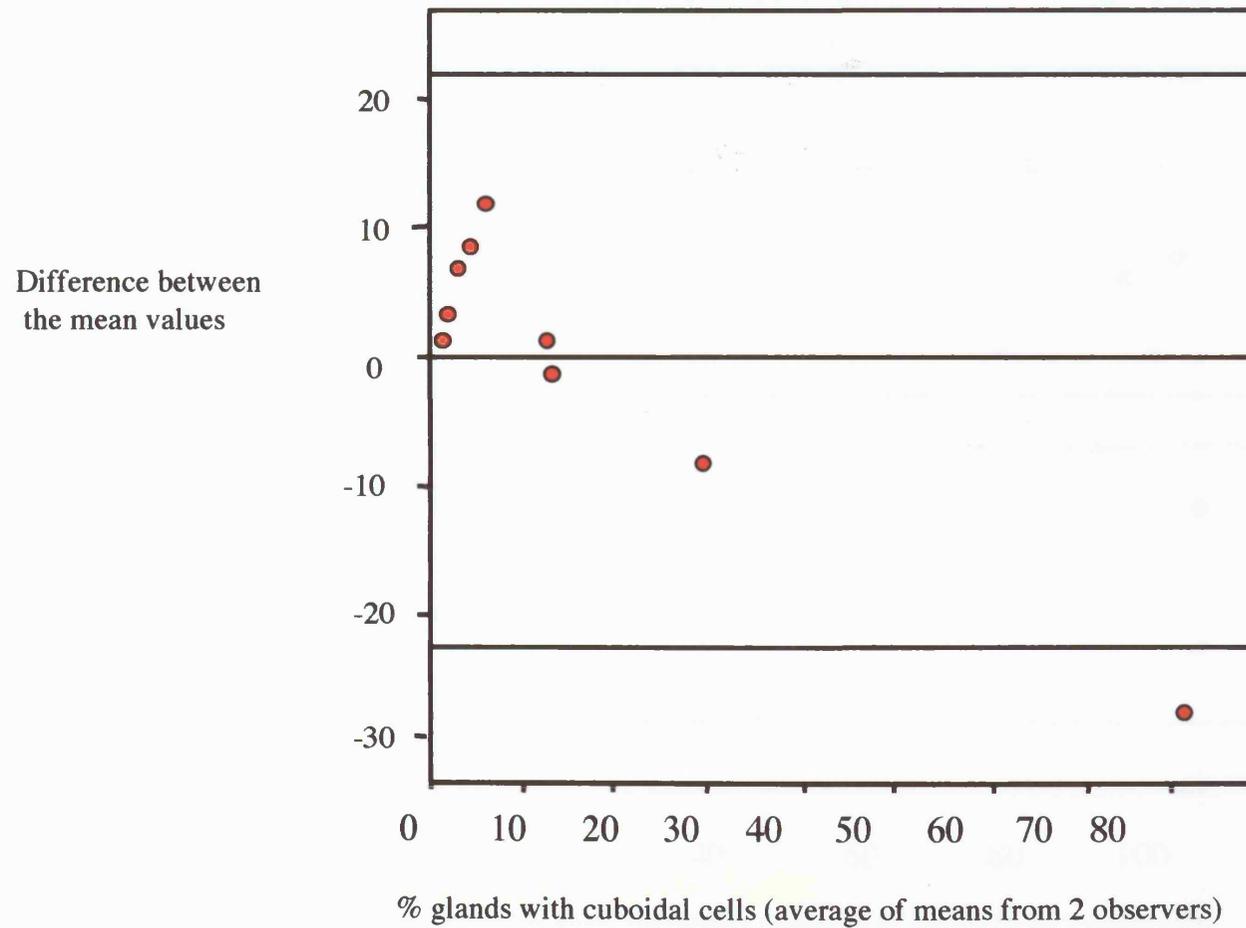


Figure 3.2c: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

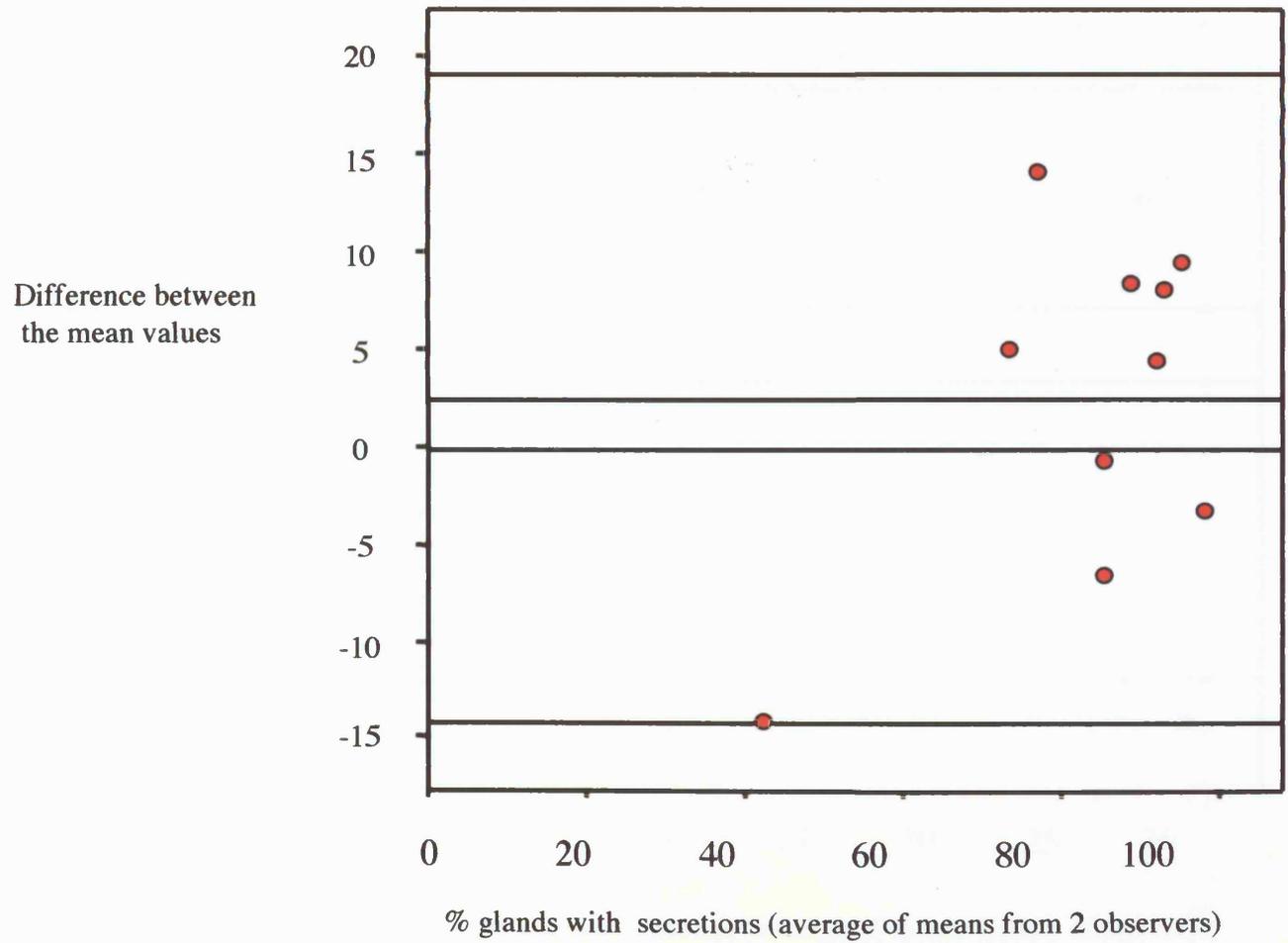


Figure 3.2d: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

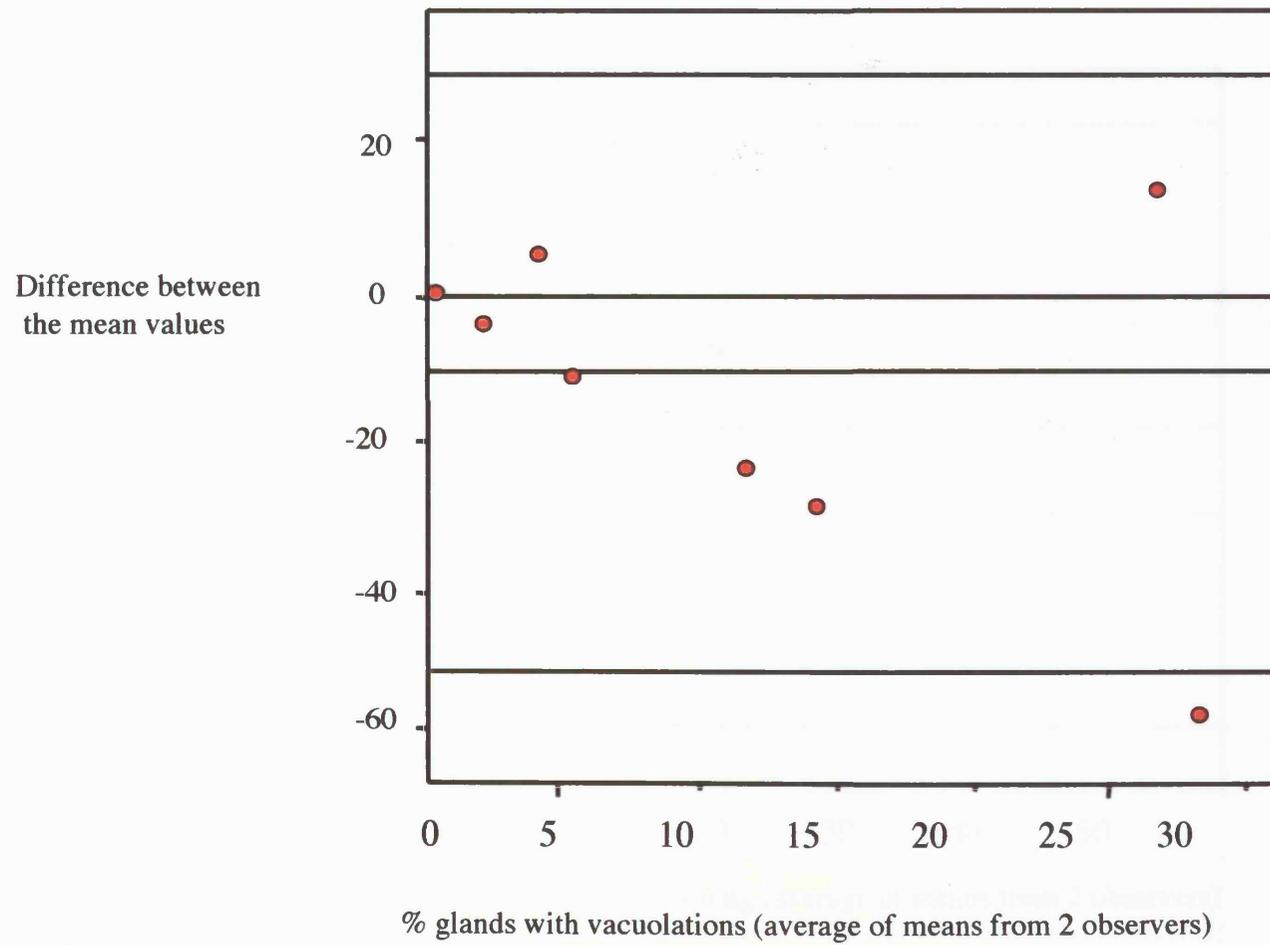


Figure 3.2e: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

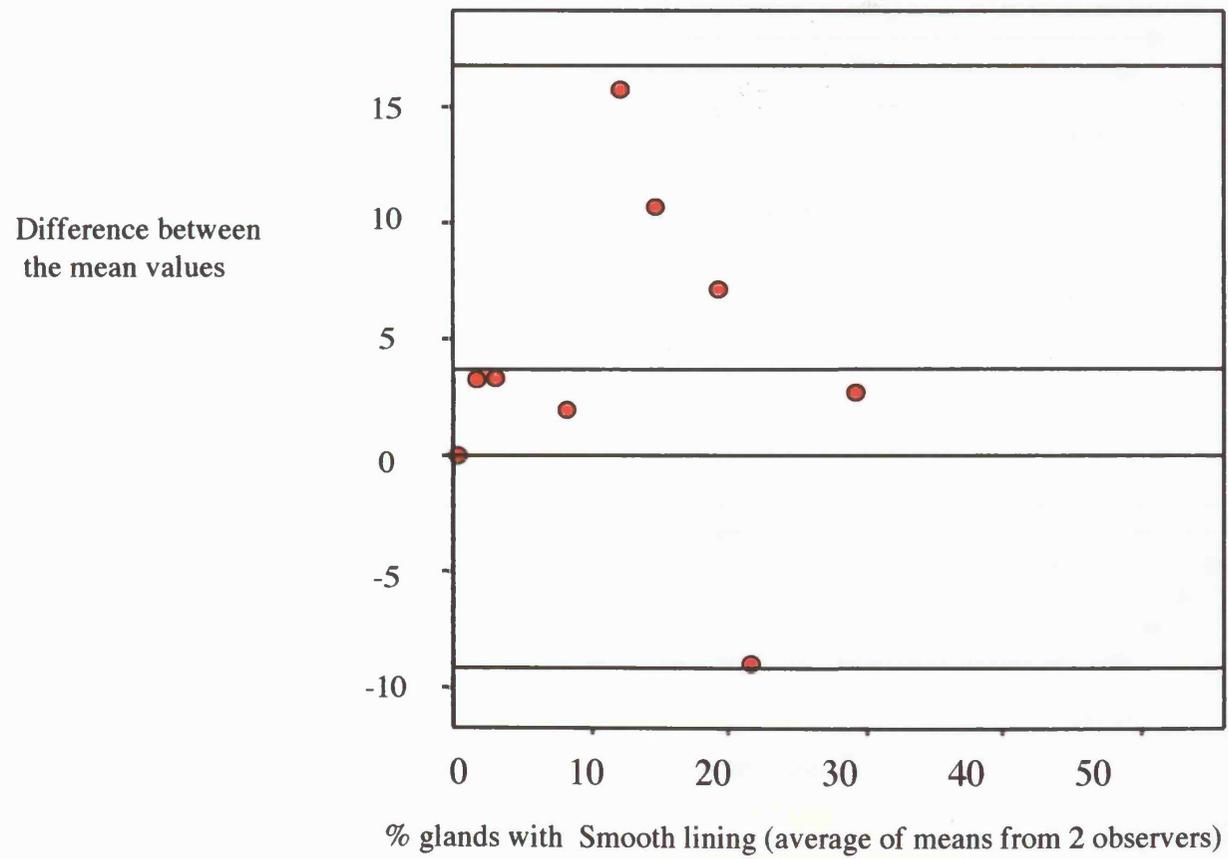
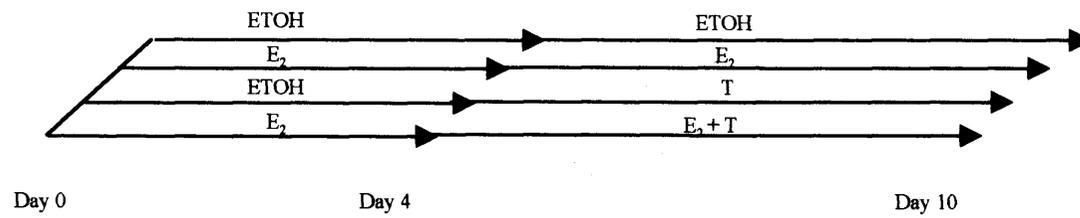
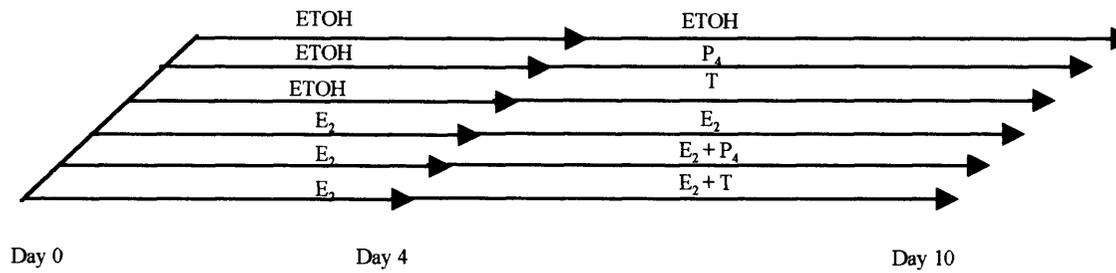


Figure 3.2f: Elimination of observers' bias: A plot of the difference between the 2 means against the average of 2 means.

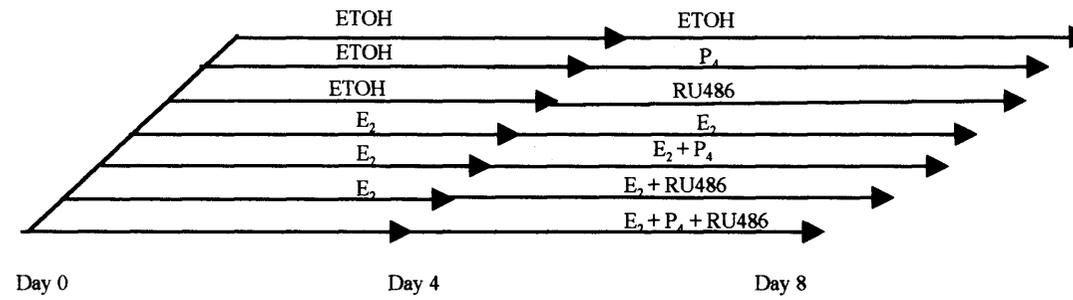


Experiment series 1

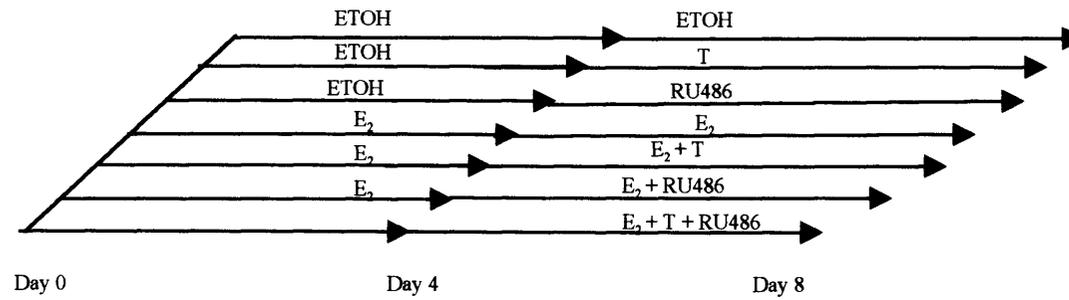


Experiment series 2

Figure 3.3a. Primary stromal cell culture. ETOH = Ethanol, E₂ = 17β-Oestradiol, P₄ = Progesterone, T = Trimegestone.



Experiment series 3a



Experiment series 3b

Figure 3.3b. Primary stromal cell culture. ETOH = Ethanol, E₂ = 17β-Oestradiol, P₄ = Progesterone, T = Trimegestone, RU486 = Mifepristone.

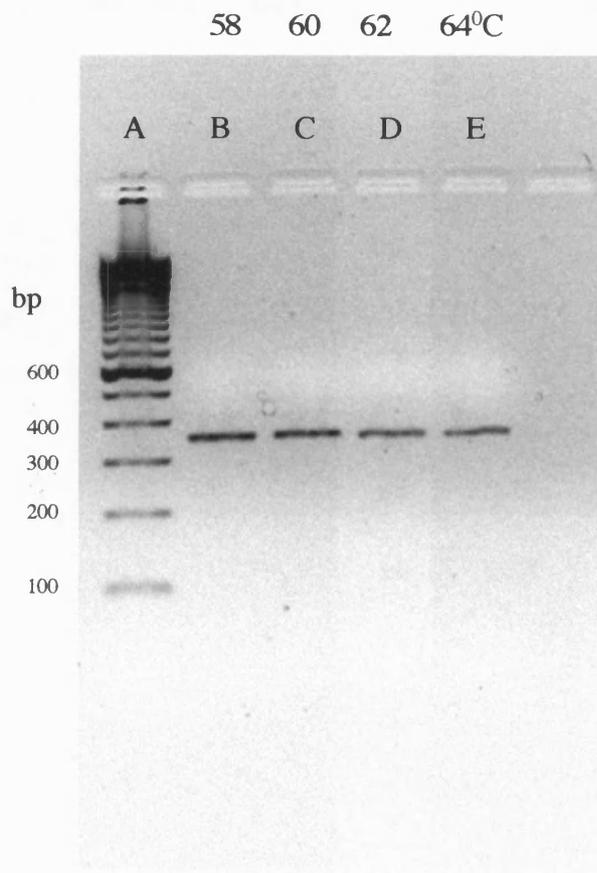


Figure 3.4a: Optimisation of GAPDH Gene amplification. Lane A= 100bp ladder. Annealing temperature at 58- 64°C (Lane B-E) for 30 cycles. GAPDH = 347bp

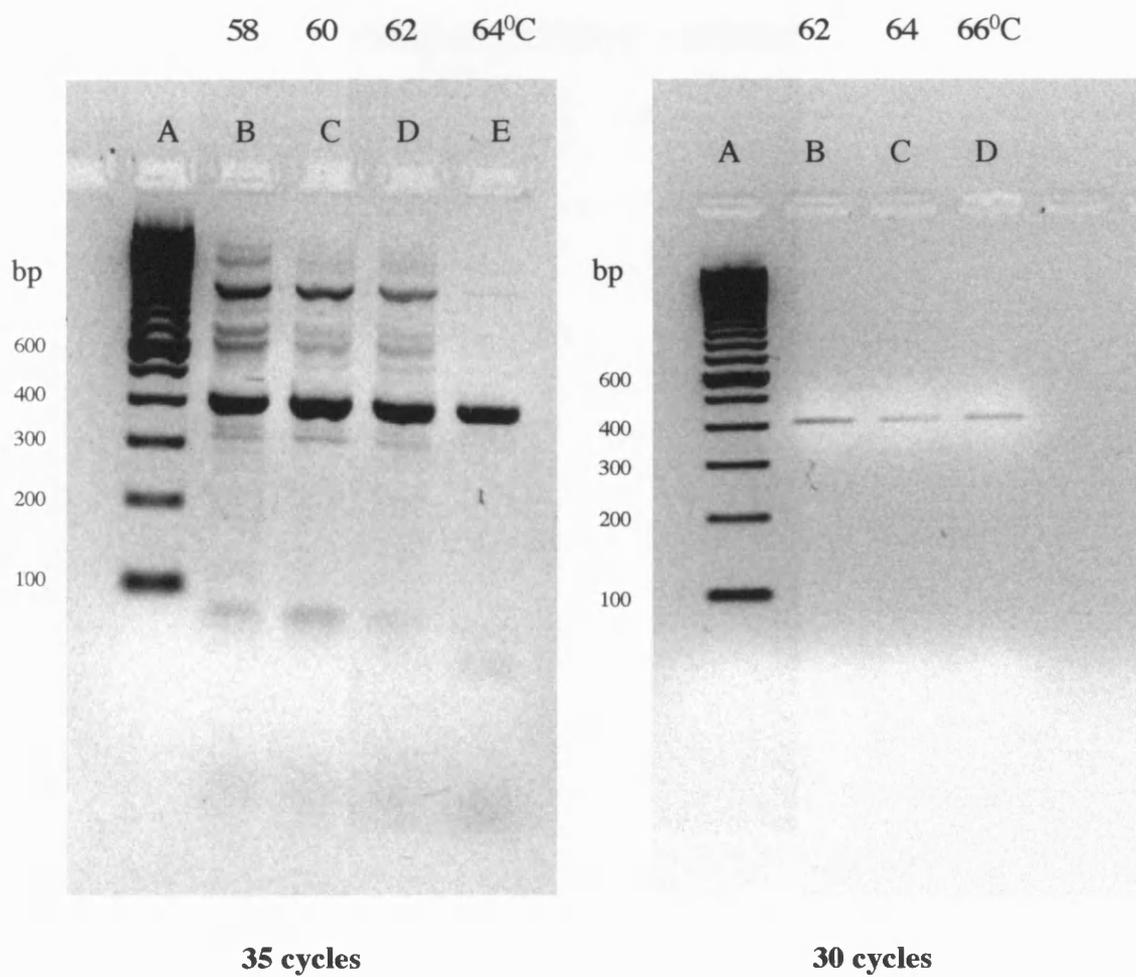


Figure 3.4b : Optimisation of MMP-1 Gene amplification. Lane A= 100bp ladder. Annealing temperature at 58 -64°C for 35 cycles (lane B-E), and 62 - 66°C for 35 cycles (lane B-D), MMP-1=381bp.

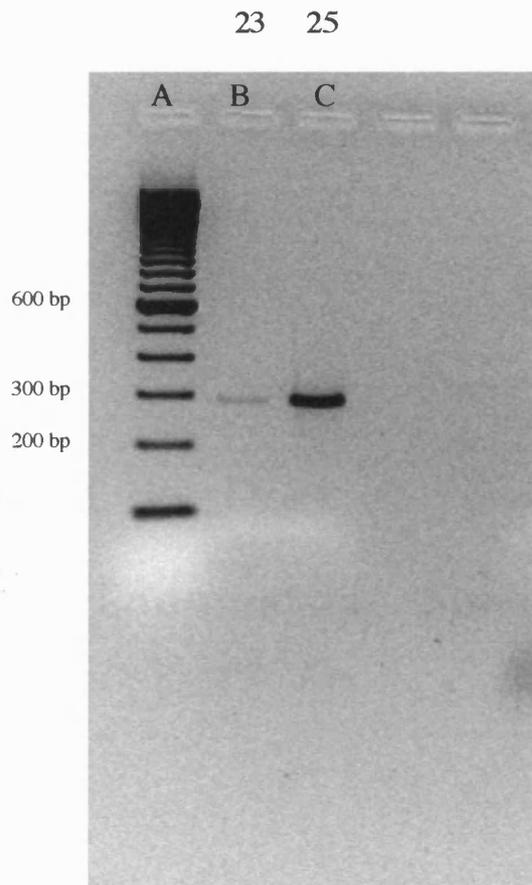


Figure 3.4c: Optimisation of MMP-3 Gene amplification. Lane A= 100bp ladder. Annealing temperature at 52^oC for 23 & 25 cycles (B & C lanes, respectively). MMP-3=266bp.

Chapter Four

Effect of the Dose of Trimegestone on Endometrial Bleeding

Introduction

The addition of cyclical progestogen to oestrogen in HRT regimens initiate withdrawal bleeding, which is the main cause for low compliance with HRT ⁵⁶. In addition, progestogens can also cause premenstrual tension (PMT) type symptoms, such as irritability, bloatedness, fluid retention and cyclical mastalgia. These PMT symptoms appear dose-related ¹⁷⁴. However, optimising the progestogen dose to below minimum effective dose may jeopardise endometrial protection and may also cause poor cycle control. Because the duration of progestogen administration appears to be an important factor in protecting the endometrium ^{175 176} shortening the exposure to the progestogen to overcome adverse events may increase the risk of hyperplasia and cancer ⁴¹.

Aim

To optimise the dose of progestogen which offered the best endometrial protection and resulted in the most acceptable pattern of bleeding.

Women and Methods

The study population with the inclusion and exclusion are described in page 51.

Treatment

The study treatment was randomly assigned to the randomisation numbers (using SAS statistical package, SAS institute Inc., Cary, NC).

The 4 doses of trimegestone were randomised on a 1:1:1:1 basis.

Women were numbered consecutively in the order in which they entered the study.

Each treatment cycle consisted of oral micronised oestradiol 2mg/day (RU 3499) for 28 days and oral trimegestone (Hoechst Marion Roussel, Romianville, France) (0.05, 0.1, 0.25, or 0.5 mg) from days 15-28. The first visit being a pre-study assessment for inclusion, 2-3 weeks prior to commencement of 168 days treatment. Women attended the unit at the end of the third cycle of treatment and on the 24th day of the sixth treatment cycle. If a woman could not attend on that day, a 7th treatment cycle was prescribed.

The second visit took place at the end of the third cycle and the third visit occurred on the 24th day of the sixth treatment cycle. Failure to attend on that day meant that a 7th cycle of treatment was prescribed.

Women completed menstrual diaries for each treatment cycle, in which the severity of the bleeding was subjectively scored. The diaries were collected at each visit and were checked for accuracy and completeness. These diaries also served as an additional parameter to check compliance since a box had to be ticked when the tablets were taken. The medication packs were collected and any remaining tablets were counted. Where less than 80% of the medications were used, non-compliance was marked and the woman withdrawn from the

study. All adverse events and concomitant medications were checked at each visit.

Diaries

Bleeding: was defined as vaginal blood loss requiring a sanitary pad or tampon. Spotting: was defined as that which did not require sanitary protection.

We defined the first day of the oestrogen phase as a fixed reference time, - "day 1". Diaries were kept for each treatment cycle;

Only completed diaries were analysed for the purpose of this study.

The bleeding data in cycles in which the endometrial biopsy was taken were excluded.

All adverse events and concomitant medications were checked at each visit.

We excluded from the bleeding analysis those cycles in which data were incomplete (incomplete diaries).

Definitions

- A bleeding episode: defined as bleeding for one or more days with at least 2 bleed-free days before and after.
- Progestogen associated bleed (PAB): vaginal bleeding which starts between day 22 of one cycle and day 7 of the next cycle, inclusive; all other bleeding were defined as intermenstrual bleeding (IMB).
- A bleeding interval; was the number of days between the first day of bleeding of two consecutive PABs.

- Total bleeding score: bleeding was scored as 0= no bleeding, 1= spotting, 2=slight bleeding, 3= moderate bleeding, 4= heavy bleeding. The total bleeding score (TBS) was the sum of the daily scores.

Statistics

Data were collected on up to 7 cycles for each woman and these repeated measures were analysed using mixed models. For continuous data, the residual maximum likelihood (REML) was used¹⁷⁷ to fit the model. For binary data, a generalised linear mixed model with binomial errors was used¹⁷⁸. Calculations were made using GENSTAT¹⁷⁹. Patient data that were not collected separately for each cycle were analysed using analysis of variance or the chi-squared test. The transition probabilities in Table 4.5 were calculated by the method of Harlow and Zeger¹⁸⁰.

Results

Between November 1994 and December 1995, 256 women were randomised to one of the 4 doses of trimegestone. Two hundred and three women completed the study of whom 202 kept diaries, and 195 provided endometrial tissue sufficient for reliable histological examination.

Women randomised into the study had an overall mean (SD) age of 51.6 years (5.9), weight of 63.8 kg (9.4), height of 161.6 cm (7.6) and body mass index of 24.4 kg/cm² (3.3). There were no statistically significant

differences between the 4 dose groups for factors of age, height, weight, body mass index (BMI), time since menopause, previous use of HRT or smoking habits.

The number of women starting and continuing treatment at each dose is shown in table 4. 1; and there was no statistically significant difference between the 4 dose groups. Table 4.2 lists the number of women who reported adverse events (AER), severe adverse events (SAE) and the number in whom the study medication was discontinued due to adverse effects (AED). The same patient can appear in all 3 categories, e.g. in the 0.05 mg/day group 36 women reported breast pain as an adverse event; 6 described it as severe and 5 withdrew as a result. There were no statistically significant differences between the numbers of patients from each trimegestone dose group for AER, SAE and AED. The following adverse events occurred and led to the discontinuation of study medications; 2 cases of thrombophlebitis, one in 0.05 mg and the other in the 0.5 mg trimegestone group. There was one case of each of the following: raised blood pressure, allergic reaction and weight loss in the 0.05, 0.1, 0.25 and 0.5 mg dose groups, respectively. Irritability and depression were contributory factors in the withdrawal of 7 women, headache of 8 women, and gastro-intestinal symptoms of 12 women, and none of these adverse events were specifically related to the trimegestone phase of treatment. One case of breast carcinoma was diagnosed during the study and therefore the woman was withdrawn. Subsequent review

revealed that the cancer had been present in the pre-treatment mammogram but had been missed.

Direct questioning on specific symptoms that might have been related to progestogen administration, namely mastalgia, acne, nausea, irritability, leg cramps, headaches, abdominal cramps, seborrhoea and bloatedness, revealed no statistically significant differences between the 4 dose groups. The total number of diaries analysed were 1104 diaries.

Frequency of PAB, IMB and amenorrhoea

These data are presented for women in Table 4.3a and for cycles in Table 4.3b. The Tables also include data on the number of cycles/women with missing data due to an incomplete diary. It should be noted that data for all women in the last treatment cycle were excluded because of the on-treatment biopsy. There were no significant differences between the 4 doses for any of these variables.

Cycle length and day of onset of bleeding

Data on the mean cycle length by dose of trimegestone and at defined times are shown in Table 4.4a. There were no significant differences between treatment groups ($P=0.44$ for all women who completed the study and $P=0.59$ for all women including those who dropped out or were withdrawn).

The mean day of onset of PAB occurred progressively later as the dose of trimegestone increased ($P<0.0001$) (Table 4.4b). I also examined the

effect of age, time since menopause, weight, BMI, smoking and dose of trimegestone on mean day of onset of PAB. Only the dose of trimegestone was a significant determinant of the differences between the groups ($P < 0.0001$). There was no change over time for either mean length of bleeding interval or mean day of onset of PAB within each dose group in those women who bled.

Analysis of the diaries on an intention to treat basis, including partial information from women who discontinued the study, showed similar results.

The Bleeding Pattern in the Individual Woman

Figure 4 shows the mean and range of day of onset of bleeding by dose of trimegestone for each woman. The mean day of onset of PAB occurred progressively later with higher doses ($P = 0.0001$). The data as illustrated suggest that the variability in the day of onset of bleeding is reduced with a mean day of onset of bleeding of 29 or later. This is more noticeable with the highest dose of trimegestone because more women on this dose bled on or after day 29. The within subject variance of the day of onset of PAB for women whose day of onset was earlier than 29 days, was significantly greater than that of women whose mean day of onset was 29 days or more ($P < 0.0001$).

Bleeding On or Before Day 28

The percentages of women who had complete diaries and who had at least one PAB starting on or before day 28 were 94%, 89%, 52% and 15%

with increasing doses of trimegestone. Similarly, 85%, 67%, 32% and 8% of all cycles started on or before day 28. These differences were highly significant ($P < 0.0001$), and the result was the same when the data were analysed on an intention to treat basis.

Prediction of the Day of Onset of PAB

I investigated whether knowledge of the day of onset of PAB in any cycle might predict the day of onset of PAB in the next cycle. Using the equation¹⁸⁰:

$$P_{ij} = \sum_{k=1}^K [F_{ijk} / F_{i.k}] / K$$

P_{ij} is the probability of transition from length category i to j .

F_{ijk} is the frequency of transition from i to j for women k .

$F_{i.k}$ is the number of times women k began in category i

K is the number of women who ever began in category i

These relationships are shown in table 4.5. For example, a day of onset of 28 days or less in the current cycle was associated with a 74% chance of this day of onset of PAB occurring in the next cycle. Absence of PAB in the current cycle was associated with a 15% chance of no PAB in the next cycle.

Number of days of PAB and IMB per cycle

The duration of PAB (in cycles with PAB and excluding cycles with no PAB) was 7.7, 7.2, 5.8, and 4.9 days with the dose of trimegestone 0.05 mg, 0.1 mg, 0.25 mg and 0.5 mg, respectively. These differences were statistically highly significant ($P < 0.0001$). Comparable data for the number of days of IMB (in cycles with IMB and excluding cycles with no IMB) were 3.4, 2.7, 1.5 and 3.4, respectively.

Total Bleeding Score (TBS)

The severity of vaginal bleeding, expressed as a TBS, progressively declined with the increase in the dose of trimegestone ($P < 0.0001$) and over time ($P < 0.0001$) (Table 4.6).

Endometrial Histology

The results of the histological assessment of the endometrium at the end of the study are shown in Table 4.7 by dose of trimegestone. There were no statistically significant differences between the treatment groups. One sample showed simple hyperplasia with secretory features in the 0.5 mg trimegestone group, and three proliferative endometria were obtained from one patient in each of the other dose groups.

Summary of the study

I assessed the effect of the dose of trimegestone in a combined cyclical sequential regimen in one of 4 doses on the pattern of bleeding in 202 postmenopausal women, who completed the study of 6 treatment cycles.

There was dominant dose effect on the pattern of bleeding. Women on the higher doses of trimegestone had later mean day of onset of PAB, tighter cycle variability, and their PAB tended to be shorter and lighter than women on the lower doses.

I tried to predict the day of onset of the PAB in the next cycle. Women who bled on or after day 29 of the HRT cycle had a higher percentage probability of bleeding on or after day 29 in the following cycle, compared to women who bled on or before day 28.

The histological assessment of the endometrial biopsy of 195 women was secretory in about 96% of women, as assessed by standard histological criteria.

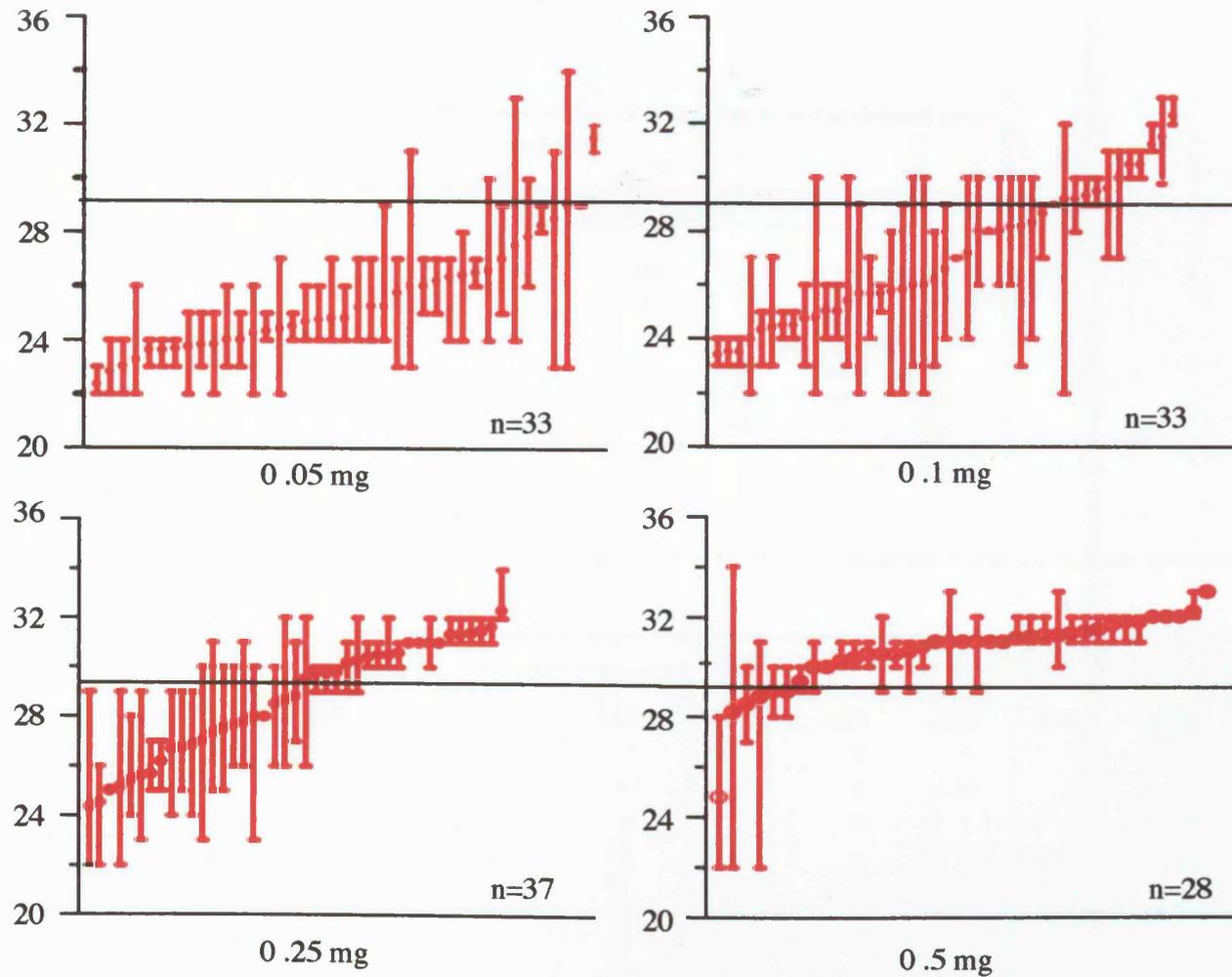


Figure 4. Mean and range of day of onset of bleeding of individual woman, by dose of trimegestone. A line drawn at day 29 separates 'early' bleeders with wide variability of the day of onset of PAB from 'late' bleeders with narrow variability.

Table 4.1. Number of women starting and continuing treatment by dose of trimegestone and at defined times. There were no statistically significant differences between the groups.

All Treated Women					
	Total	0.05	0.1	0.25	0.5
Total No of Women	266	66	72	68	60
No. of women receiving treatment					
Cycle 1- Cycle 3	258	62	69	68	59
Cycle 4- Cycle 5	214	50	56	58	50
Cycle 6- Cycle 7	200	48	51	55	46

Table 4.2. Number of women reporting adverse events (AER), severe adverse events (SAE), and the number in whom study medication was withdrawn (AED) by dose of trimegestone.

	0.05 mg			0.1 mg			0.25 mg			0.5 mg		
	AER	SAE	AED	AER	SAE	AED	AER	SAE	AED	AER	SAE	AED
Menstrual Problems	5	3	4	3	2	2	4	3	3	3	1	2
Breast Pain	36	6	5	41	9	4	34	11	3	25	8	2
Acne	5	2	1	4	0	0	1	0	0	5	0	0
Seborrhoea	5	1	1	2	0	0	0	0	0	1	0	0
Bloatedness	32	4	1	31	3	3	30	8	1	30	10	3

Table 4.3a & 3b The number of women(3a) and number of cycles (3b) with biopsies, missing data, progestogen associated bleeding (PAB), inter menstrual bleeding (IMB), both PAB and IMB and amenorrhoea in study completers.

	0.05 mg	0.10 mg	0.25 mg	0.50 mg	Total
3 a					
Women	48	54	54	47	203
Women with some missing data due to biopsy	47	46	52	44	189
Women with some missing data due to incomplete diaries	15	10	19	12	56
Women with at least one PAB only cycle	48	54	54	46	202
Women with at least one IMB only cycle	2	0	1	4	7
Women with at least one PAB and IMB cycle	3	9	4	4	20
Women with at least one amenorrhoeic cycle	0	4	4	2	10
3 b					
Cycles	268	287	294	255	1104
Cycles missing due to biopsy	59	55	64	51	229
Cycles missing due to incomplete data	20	10	25	19	74
Cycles with PAB only	180	207	194	174	755
Cycles with IMB only	4	0	1	5	10
Cycles with PAB and IMB	5	10	5	4	24
Amenorrhoeic Cycles	0	5	5	2	12

Table 4.4a & 4b. The mean length of bleeding interval at defined times and by dose of trimegestone (4a) and mean day of onset of PAB at defined times and by dose of trimegestone (4b).

Table 4.4a Mean cycle length

Cycle	0.05 mg		0.10 mg		0.25 mg		0.50 mg		
	No.	n	mean	n	mean	n	mean	n	mean
1-2	41	41	27.5	47	27.7	41	27.3	35	28.2
2-3	41	41	27.7	46	28.1	41	28.3	35	27.9
3-4	41	41	28.1	45	27.7	43	27.7	39	28.0
4-5	40	40	28.1	49	27.8	44	28.7	40	28.1
All	163	163	27.9	187	27.8	169	28.0	149	28.1

Table 4.4b Day of onset of cyclical bleeding

Cycle	0.05 mg		0.10 mg		0.25 mg		0.50 mg		
	No.	n	mean	n	mean	n	mean	n	mean
1	43	43	25.0	54	25.3	48	29.5	41	29.9
2	45	45	24.6	50	25.7	51	26.5	41	29.2
3	45	45	24.8	50	25.6	46	29.2	42	28.5
4	44	44	24.5	50	25.4	49	27.5	43	30.6
5	47	47	23.4	50	25.8	50	29.1	44	29.3
All	224	224	24.5	254	25.6	244	28.3	211	29.5

Table 4.5. Percentage probability of the day of onset of the Progestogen associated bleeding (PAB) in the current cycle predicting this in the next cycle. All doses of trimegestone combined.

Current cycle	Next cycle			
	No bleeding	≤ 28	29-31	≥ 32
No bleeding	15	29	45	11
≤ 28	2	74	21	3
29-31	2	28	56	14
≥ 32	12	6	39	43

Table 4.6. Mean of Total Bleeding Score (PAB) during progestogen associated bleeding (PAB) by trimegestone dose and at defined times during the study.

Cycle	0.05 mg		0.10 mg		0.25 mg		0.50 mg	
	No.	mean	n	mean	n	mean	n	mean
1	43	19.3	54	16.7	48	12.9	41	12.6
2	45	16.5	50	16.5	51	11.9	41	10.6
3	45	16.8	50	15.2	46	11.8	42	10.4
4	44	15.6	50	15.0	49	11.7	43	10.0
5	47	14.8	50	14.7	50	10.9	44	9.6
All	224		254		244		211	

Table 4. 7. Histological findings at the end of the study by dose of trimegestone.

	Total	0.05 mg	0.1 mg	0.25 mg	0.5 mg
Total No of treated women	266	66	72	68	60
No. Evaluable women	195	47	54	49	45
Endometrial biopsy n(%)					
Inactive	1 (0.5)	0	0	1 (2)	0
Early secretory	7 (3.6)	1 (2.1)	1 (1.9)	3 (6.1)	2 (4.4)
Late secretory	137 (70.3)	27 (57.4)	38 (70.4)	38 (77.6)	34 (75.6)
Retarded endometrium atrophic late secretory (HRT effect)	11 (5.6)	0	4 (7.4)	2 (4.1)	5 (11.1)
Late secretory (menstrual)	31 (15.9)	16 (34)	9 (16.7)	4 (8.2)	2 (4.4)
Proliferative	3 (1.5)	1 (2.1)	1 (2.0)	1 (2.0)	0
Simple hyperplasia	1 (0.5)	0	0	0	1 (2.2)
Inadequate tissue but atrophic by hysteroscopy	4 (2.1)	2 (4.3)	1 (1.9)	0	1 (2.2)

Chapter Five

Effect of the Dose and Duration of

Trimegestone

on Endometrial Bleeding

Introduction

In chapter 4, I have illustrated a clear dose-dependent pattern of endometrial bleeding in postmenopausal women treated with trimegestone-based sequential combined HRT.

A link between inadequate endometrial secretory changes and the occurrence of bleeding before the end of the progestogenic phase in sequential combined HRT has been suggested ¹⁸¹. This observation was challenged by a larger study of women receiving different HRT regimens ¹⁸². Sturdee and co-workers (1994) found no correlation between the day of onset of bleeding and endometrial histology among 413 women who had used sequential combined HRT regimens for 3 months. This concurred with the findings in trimegestone dose ranging study (Chapter 4), where despite the widely variable day of onset of bleeding; about 96% of endometrial samples had secretory changes.

However, while Padwick et al. (1986) proposed the need to increase the dose of progestogen to improve endometrial histology and delay the day of onset of bleeding, they did not show their results in a subsequent publication. Nevertheless, increasing the dose of progestogen in women experiencing unscheduled bleeding when received continuous combined HRT resulted in reduction of the bleeding episodes ¹⁸³.

However, the increasing incidence of progestogenic and androgenic adverse events upon raising the dose of progestogen is one of the limitations in clinical practice.

Aims

- a. To test the hypothesis that the pattern of bleeding is highly dependent on the dose of trimegestone in the sequential combined HRT regimen, I analysed the menstrual diaries of women participated in the original dose ranging study and who continued the treatment for a further 6 months with a change in the dose of trimegestone to 0.25 mg.
- b. The effect of the duration of use on the pattern of bleeding in women who did not change the dose of trimegestone.

Women and methods

The study population with the inclusion and exclusion are described in page 51.

Treatment

Each cycle consisted of oral micronised oestradiol (RU 3499: Hoechst Marion Roussel; Romainville, France) 2 mg/day for 28 days and one dose of 0.25 mg per day of trimegestone tablets from day 15-28 of the treatment cycle, for 6 cycles.

Women completed menstrual diaries for each treatment cycle as described in chapter 4.

Definitions of bleedings events

These are described on page 91.

Statistics

The statistical method used in this extension study are described in page 92.

Results

The extension phase of the original dose ranging study included 134 women who elected to continue the study, and received one dose of trimegestone (0.25mg), for a further 6 treatment cycles of whom 107 completed the study (Table 5.1).

Women randomised into the study had an overall mean (SD) age of 52.7 years (5.4), weight of 65.3 kg (9.5), height of 161.5 cm (6.7). The body mass index was 25.0 kg /cm² (3.3), which compares with mean BMI of postmenopausal women in UK ¹⁸⁴. There were no statistically significant differences between the treatment groups in terms of age, race, height, weight, body mass index and smoking habits.

Twenty seven women did not complete the second 6 months of the study due to; adverse events (12): protocol violation (2) women's request (6) and lost to follow up (4). There were no statistically significant differences between the numbers of patients from each trimegestone dose group for adverse events (AER), severe adverse events (SAE) and number of women in whom study medication was withdrawn (AED), nor was there a statistically significant difference between the number of women who withdrew from each trimegestone dose group. Table 5.2 shows the number of women with AER, SAE and AED. Some women may appear in all 3 categories. There were 3 serious adverse events necessitating medical investigation and the women

withdrew from the study; myocardial ischaemia, facial paralysis and gastro-intestinal complaint.

Six hundred and twenty eight menstrual diaries were analysed. Only completed diaries were evaluated and to the day of withdrawal for women who did not complete the study.

Mean day of onset of the PAB (fig. 5. 1 a & b)

The mean day of onset of PAB in the first 6 cycles in women on the lower doses of 0.05 and 0.1 mg were 25.2 and 26.5 respectively, this changed in the following 6 cycles and occurred later on days 28.8 and 29 ($P = 0.0001$ and $P = 0.0001$, respectively, Table 5.3). Women who continued on the 0.25 mg trimegestone showed no change in the mean day of onset of the PAB. However women on the 0.5 mg dose of trimegestone who had a mean day of onset of the PAB on 30.9 in the first 6 cycles, now had an earlier mean day of onset of the PAB of 29 ($P = 0.0001$).

Duration of the PAB (Fig. 5. 2)

Women on the lower doses of trimegestone 0.05 and 0.1 mg had shorter PAB when given the higher dose of 0.25 mg, from 7.01 and 6.48 to 5.7 ($P = 0.0001$) and 5.7 ($P = 0.001$), respectively, Table 5.4. Women who continued on 0.25 mg dose experienced no change in the PAB duration, while women who bled for only 4.6 days on the 0.5 mg dose during the first 6 months, now had a prolonged bleeding of 5.4 days on changing to the lower dose ($P = 0.001$).

Severity of the PAB (TBS, Fig. 5. 3)

Women on the trimegestone doses of 0.05 and 0.1 mg who had mean TBS of 15.5, 14.3 in the first 6 cycles, experienced lighter bleeding in the second six months, when received the 0.25 mg of trimegestone, of 11.8 and 12.6 ($P = 0.0001$ and $P = 0.001$ respectively, Table 5.5). Women who continued on the 0.25 mg dose experienced no change in the severity of the bleeding (11.6 and 11.4). Similarly, there was no significant change in the severity of bleeding in women on the 0.5 mg dose after changing to the lower dose of 0.25 mg (TBS = 10.6 and 11.5).

Incidence of the IMB

There was no difference in the incidence of IMB in the lower dose groups, however there was 3 folds increase in the incidence of IMB in the 0.25mg dose group, when they continued on the same dose for a further 6 cycles, Table 5.6. In the 0.5 mg dose group there was significant reduction in the incidence of IMB from 10 women in the first 6 months, to only 1 in the following 6 months.

Histology

The histological assessment of the endometrium at the end of the extension study showed secretory changes (95.4%), proliferative changes (1.5%), inactive endometrium (0.8%), atrophic (2.3%, table 5.7).

Summary of the study

One hundred and thirty four postmenopausal women continued for a further 6 cycles on one dose of trimegestone (0.25mg), combined with oestradiol in a cyclical sequential regimen. There was no dose effect on the incidence of progestogenic or androgenic adverse events. The results confirmed that the dose of trimegestone was the major determinant of the pattern of bleeding. Women treated with 0.05 and 0.1mg trimegestone in the first 6 months now experienced a later mean day of onset of bleeding which was lighter and of shorter duration. Women who were on the 0.5 mg dose in the first 6 months; regressed to experience earlier onset of heavier and more prolonged bleeding during the extension phase of the study. However, there was no improvement in the pattern of bleeding in women who continued on the 0.25 mg dose.

The histological assessment of endometrial samples at the end of the extension study showed secretory changes in about 96% of women.

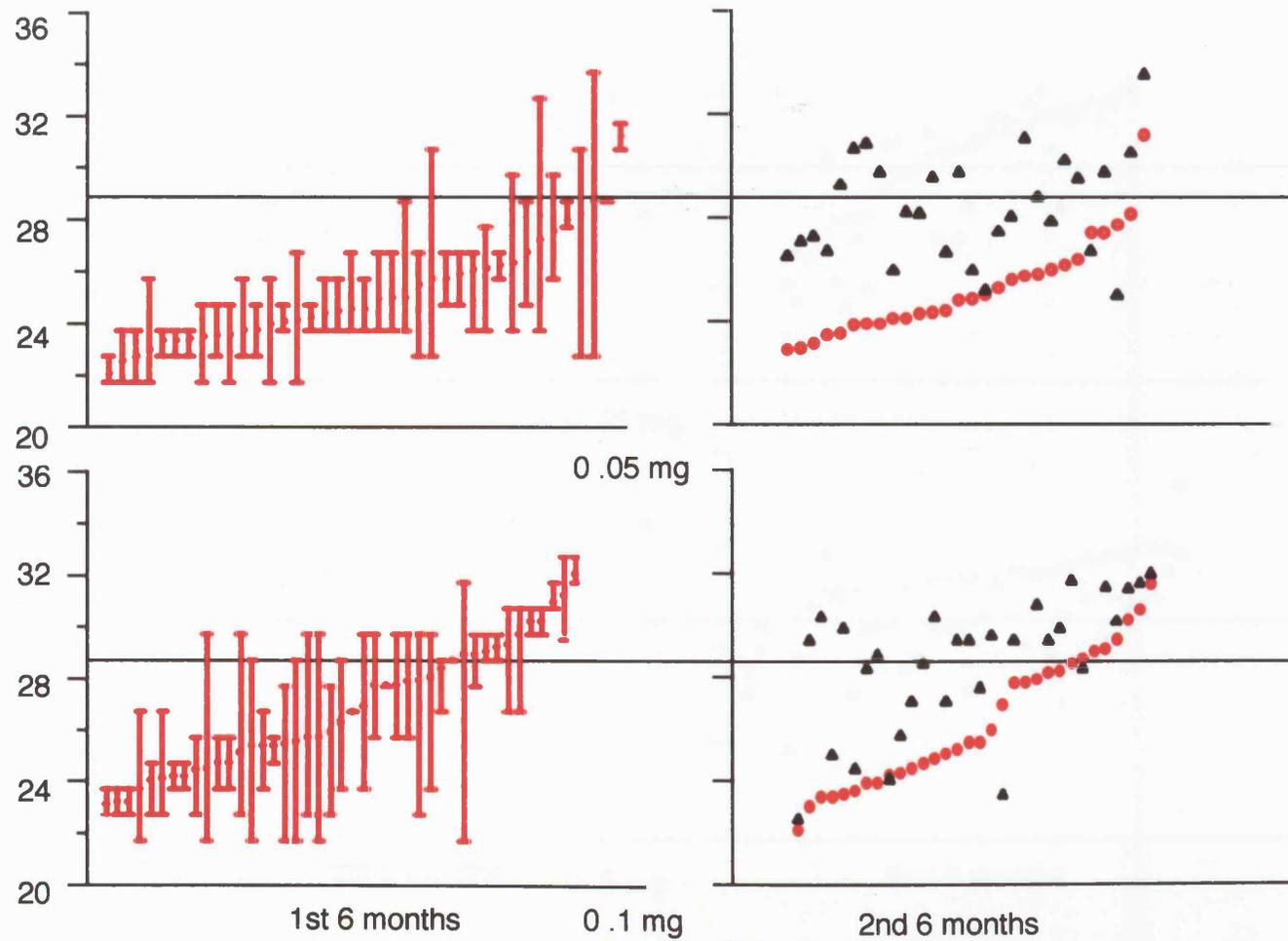


Fig.5.1a. Mean day of onset of the progestogen associated bleeding (PAB) in the 0.05 and 0.1mg in the first 6 months compared to the second 6 months. Circles are mean day of onset of the PAB in individual woman in the 1st 6 months, triangles are mean day of onset of the PAB in individual woman in the second 6 months.

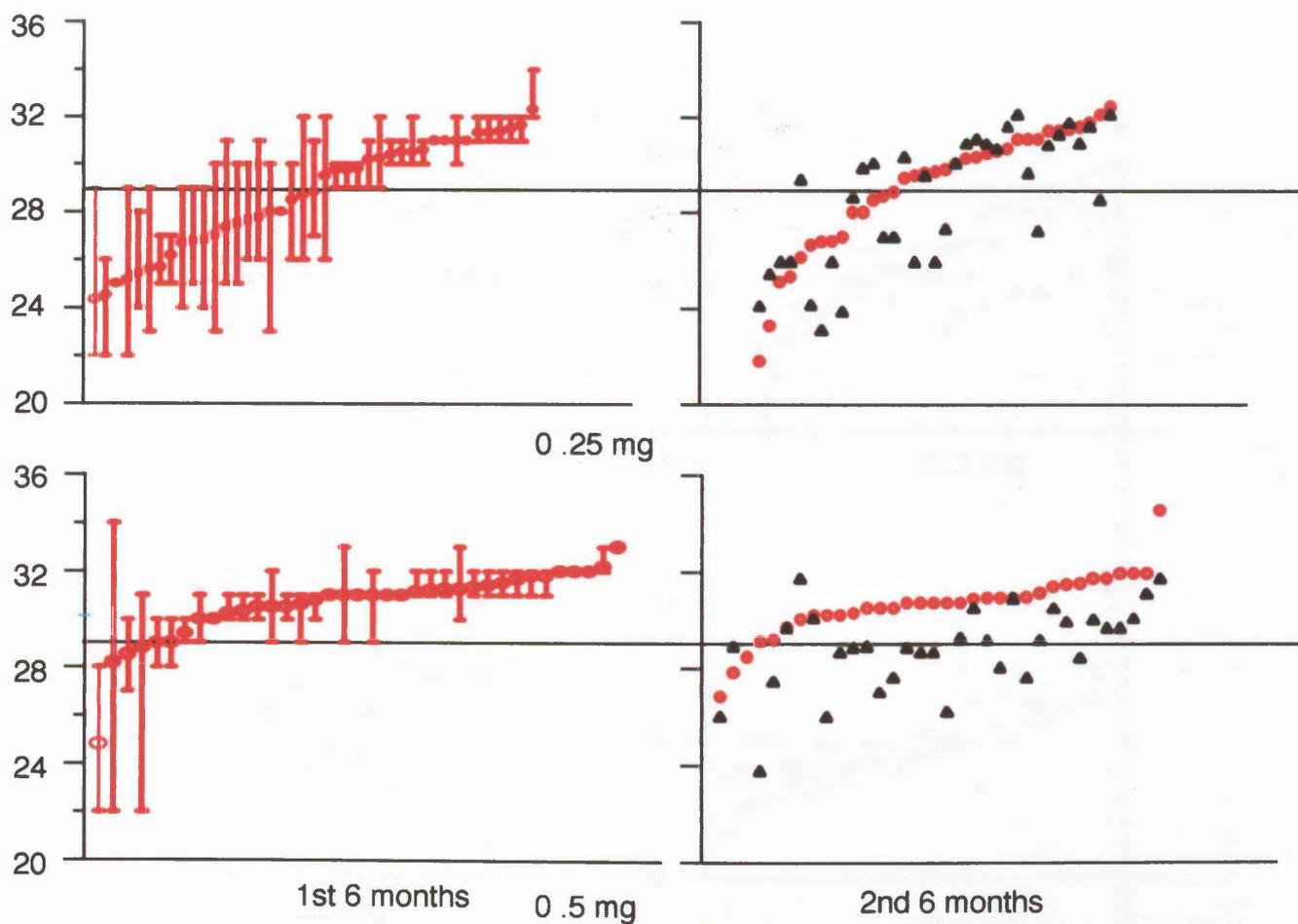


Fig. 5.1b.: Mean day of onset of the progestogen associated bleeding (PAB) in the 0.25 & 0.5mg in the first 6 months compared to the second 6 months. Circles are mean day of onset of the PAB in individual woman in the 1st 6 months, triangles are mean day of onset of the PAB in individual woman in the second 6 months.

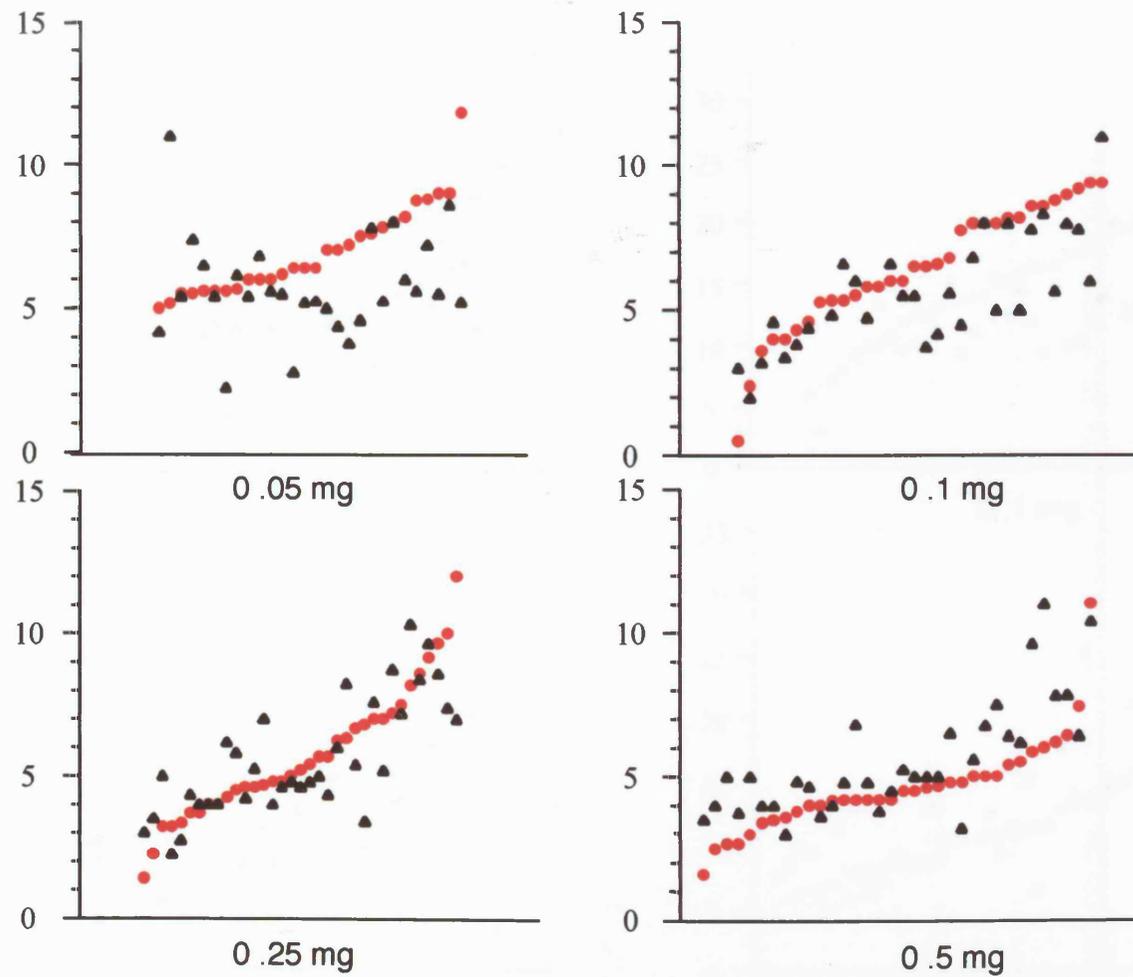


Fig. 5. 2. Duration of the progestogen associated bleeding (PAB) in the 4 dose groups, 0.05, 0.1, 0.25 & 0.5mg in the first 6 months compared to the second 6 months. Circles are duration of the PAB in individual woman in the 1st 6 months, triangles are duration of the PAB in individual woman in the second 6 months.

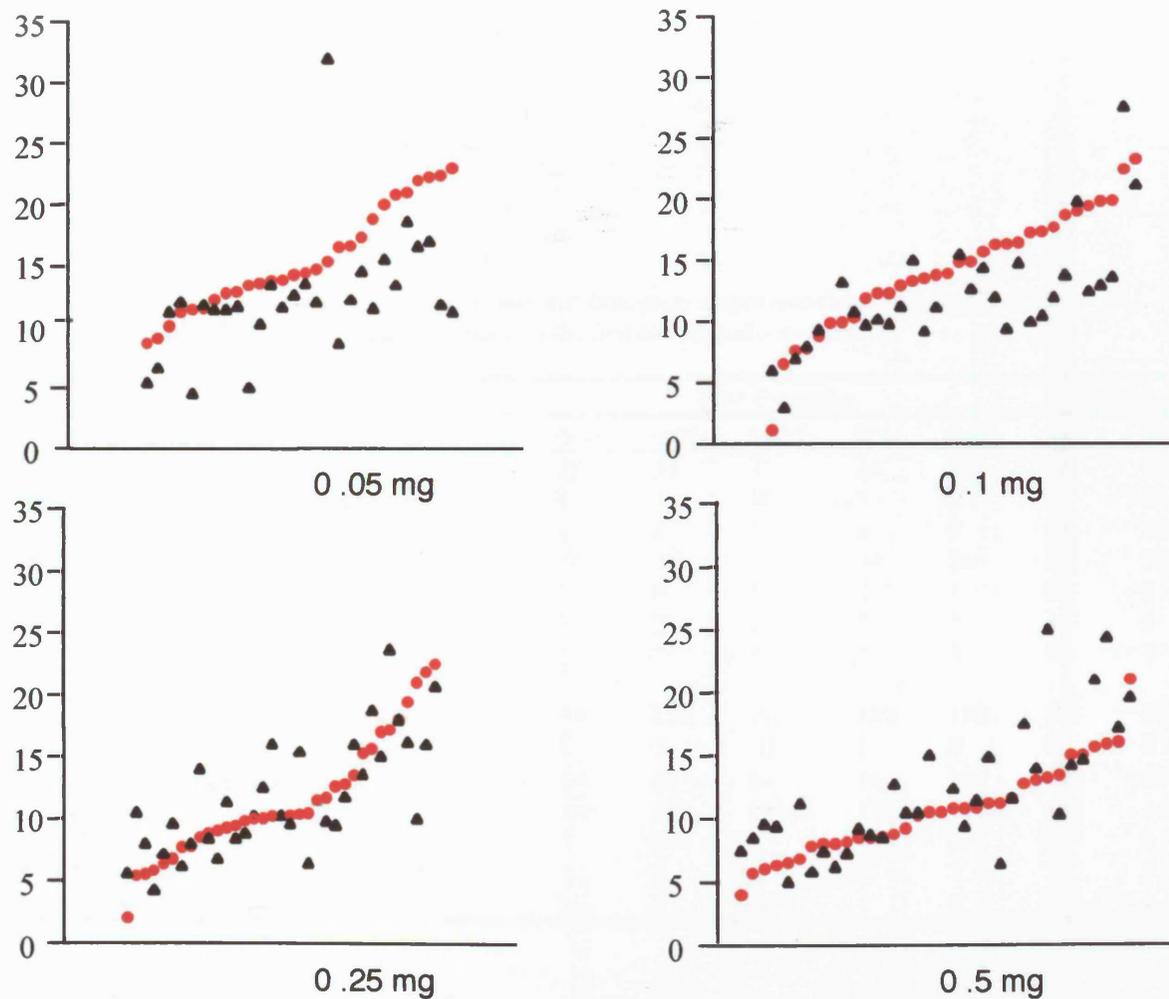


Fig. 5.3 Total bleeding score (TBS) of the progestogen associated bleeding (PAB) in the 4 dose groups, 0.05, 0.1, 0.25 & 0.5mg in the first 6 months compared to the second 6 months. Circles are TBS of the PAB in individual woman in the 1st 6 months, triangles are TBS of the PAB in individual woman in the second 6 months.

Table 5.1 Number of women and the cycles with biopsies, missing data, progestogen associated bleeding (PAB), Intermenstrual bleeding (IMB), PAB+IMB and amenorrhoea, in the first and second 6 months.

	First 6 months				Second 6 months			
	0.05	0.01	0.025	0.5	0.05	0.01	0.025	0.5
No of women	28	32	35	34	28	32	35	34
Women with some missing data due to biopsy	8	7	10	5	0	0	0	0
Women with some missing data due to incomplete diaries	11	8	11	9	9	13	16	12
Women with at least one PAB only cycle	28	32	35	34	28	30	35	34
Women with at least one IMB only cycle	1	0	1	2	1	0	1	0
Women with at least one PAB and IMB cycle	2	2	1	5	3	3	5	1
Women with at least one amenorrhoeic cycle	2	2	2	1	3	3	5	1
No of cycles	157	175	197	189	140	147	186	155
Cycles missing due to biopsy	8	7	10	5	0	0	0	0
Cycles missing due to incomplete data	14	10	14	16	10	18	27	14
Cycles with PAB only	128	151	168	156	122	121	149	136
Cycles with IMB only	3	0	1	4	2	0	1	0
Cycles with PAB and IMB	2	4	1	7	3	7	5	1
Amenorrhoeic Cycles	2	3	3	1	3	1	4	4

Table 5.2. Number of women reporting progestogenic adverse events associated with study treatment (AER), severe adverse events (SAE), and the number in whom study medication was withdrawn (AED) by dose of trimegestone.

	0.05 mg			0.1 mg			0.25 mg			0.5 mg		
	AER	SAE	AED	AER	SAE	AED	AER	SAE	AED	AER	SAE	AED
		n=66		n=72			n=68			n=60		
Menstrual Problems	0	0	0	1	0	0	1	1	1	1	0	0
Breast Pain	12	4	1	14	2	0	17	5	1	11	1	0
Acne	0	0	0	2	0	0	0	0	0	5	0	0
Bloatedness	10	0	0	11	1	0	15	1	0	13	2	1

Table 5.3 Mean day of onset of progestogen associated bleeding (PAB).

First 6 months		0.05		0.1		0.25		0.5	
Cycles	No	mean	No	mean	No	mean	No	mean	
1-2	25	24.8	29	26.6	31	29.6	29	30.9	
2-3	24	25.5	29	26.7	29	28.4	28	30.9	
3-4	26	25.2	30	26.6	31	29.3	30	31.0	
4-5	23	25.4	29	26.4	32	28.6	32	30.7	
5-6	24	25.4	29	26.4	33	29.6	33	30.8	
Total	122	25.2	146	26.4	156	29.1	152	30.9	
Second 6 months		0.05		0.1		0.25		0.5	
Cycles	No	mean	No	mean	No	mean	No	mean	
1-2	23	29.4	28	29.3	30	28.9	31	28.3	
2-3	27	28.3	28	28.6	32	28.6	32	28.9	
3-4	24	29	22	30.0	29	28.7	24	28.8	
4-5	25	29.1	22	28.5	29	28.5	22	29.3	
5-6	23	28.4	22	28.9	29	28.3	24	30.1	
Total	122	28.8	122	29.0	149	28.6	133	29.0	

Table 5.4 The duration of the progestogen associated bleeding (PAB).

First 6 months	0.05		0.1		0.25		0.5	
Cycles	No	mean	No	mean	No	mean	No	mean
1-2	25	7.96	30	6.66	31	5.58	30	5.07
2-3	24	6.96	29	6.86	32	5.97	30	4.67
3-4	26	7.39	31	6.26	31	5.65	31	4.58
4-5	24	6.83	30	6.27	33	5.88	32	4.44
5-6	27	5.96	29	6.45	33	5.27	33	4.36
Total	126	7.01	149	6.48	160	5.27	156	4.62
Second 6 months								
Cycles	No	mean	No	mean	No	mean	No	mean
1-2	24	5.67	28	5.89	31	5.74	31	5.97
2-3	28	5.89	28	5.54	32	5.75	32	5.91
3-4	24	6.04	23	5.39	30	5.57	26	4.92
4-5	26	5.35	22	6.14	31	5.29	24	5.13
5-6	24	5.54	22	5.68	30	5.63	24	5.21
Total	126	5.7	123	5.27	154	5.6	137	5.47

Table 5.5 The total bleeding score (TBS) during progestogen associated bleeding (PAB).

First 6 months	0.05		0.1		0.25		0.5	
Cycles	No	mean	No	mean	No	mean	No	mean
1-2	25	18.5	30	15.4	31	12.2	30	12.2
2-3	24	15.5	29	15.5	32	21.1	30	10.7
3-4	26	16.3	31	13.5	31	11.8	31	10.5
4-5	24	14.1	30	13.5	33	11.6	32	10.1
5-6	27	13.2	29	13.7	33	10.2	33	9.5
Total	126	15.5	149	14.3	160	11.6	156	10.6
Second 6 months								
Cycles	No	mean	No	mean	No	mean	No	mean
1-2	24	13.2	28	13.6	31	13.3	31	12.9
2-3	28	12.0	28	12.2	32	11.7	32	12.1
3-4	24	12.7	23	11.6	30	10.9	26	10.2
4-5	26	11.0	22	13.4	31	10.4	24	10.8
5-6	24	10.4	22	12.1	30	10.9	24	11.1
Total	126	11.8	123	12.6	154	11.4	137	11.5

Table 5.6 Mean No. of Intermenstrual bleeding (IMB) episodes.

First 6 months		0.05		0.1		0.25		0.5	
Cycles	No	mean	No	mean	No	mean	No	mean	
1-2	0		0		0		4	2	
2-3	1	1	1	3	1	4	3	3.7	
3-4	0		2	1.5	0	0	2	4.5	
4-5	1	6	1	3	1	1	0		
5-6	1	8	0		0		1	1	
Total	3	5	4	2.3	2	2.5	10	2.9	
Second 6 months									
Cycles	No	mean	No	mean	No	mean	No	mean	
1-2	1	3	2	2	2	2.5	0		
2-3	1	3	1	1	0		0		
3-4	1	1	1	1	1	1	1	1	
4-5	1	1	2	1.5	2	2	0		
5-6	0		0		1	2	0		
Total	4	2	6	1.5	6	2	1	1	

Table 5.7. Histological findings at the end of the study (12 months) by dose of trimegestone.

	Total	0.05 mg	0.1 mg	0.25 mg	0.5 mg
Total No of treated women	134	29	32	38	35
No. Evaluable women	130	29	31	35	35
Endometrial biopsy n(%)					
Inactive	1 (0.8)	0	0	1 (2.9)	0
Early secretory	4 (3.1)	0	0	3 (8.6)	1 (2.9)
Late secretory	93 (71.5)	16 (55.2)	23 (74.2)	25 (71.4)	29 (82.9)
Retarded endometrium atrophic late secretory (HRT effect)	6 (4.6)	0	2 (6.5)	2 (5.7)	2 (5.7)
Late secretory (menstrual)	21 (16.2)	11 (37.9)	4 (12.9)	4 (11.4)	2 (5.7)
Proliferative	2 (1.5)	1 (3.4)	1 (3.2)	0	0
Inadequate tissue but atrophic by hysteroscopy	3 (2.3)	1 (3.4)	1 (3.2)	0	1 (2.9)

Chapter Six

Endometrial Structural Abnormalities

Introduction

Endometrial structural abnormalities in postmenopausal women are associated with unscheduled uterine bleeding while on HRT^{104 106}. Of all fibroids, the submucous type seems to be the most symptomatic¹⁸⁵. Over 45% of women with irregular or heavy bleeding on HRT had structural lesions on hysteroscopic assessment^{105 106}.

Aim

To assess the influence of endometrial structural abnormalities on the pattern of endometrial bleeding in women treated with sequential trimegestone combined with micronised oestradiol 2 mg daily in a dose ranging study (4 doses) for 6 treatment cycles.

Women and Methods

One hundred and seventy six women were subgroup of the total study population of 256 studied in Chapter 4 (Page 52). The inclusion and exclusion criteria are described in page 51. Women underwent diagnostic hysteroscopy as an out-patient procedure under local anaesthesia, at baseline and at the end of the study. Submucous fibroids and endometrial polyps were differentiated by their appearance and consistency.

Submucous fibroids were graded according to the degree of protrusion into the endometrial cavity into 3 grades: I, II and III, depending on how much of an estimated sphere was protruding into the endometrial cavity: up to 1/3, up to 2/3 or >2/3, respectively. The endometrium was

sampled using the Vabra curette and women with evidence of hyperplasia or carcinoma were excluded.

Treatment

Women were randomised to receive one of 4 dose of trimegestone as described on page 90.

Definitions

- Bleeding episode was defined as an episode of uterine bleeding which lasts for one or more days with at least one clear day before and after.
- Progestogen associated bleeding (PAB) was defined as the longest bleeding episode, which starts between day 22 of one cycle and day 7 of the next treatment cycle inclusive.
- Intermenstrual bleeding; all other bleeding episodes were called (IMB).

Statistics

Data were collected on up to 7 treatment cycles and were analysed using mixed effects regression models. For continuous data, such as day of onset of the PAB, duration and severity of the bleeding, the residual maximum likelihood was used to fit the model ¹⁷⁷. For binary data, such the occurrence of IMB, a generalised linear mixed model with binomial errors was used ¹⁷⁸. Calculations were made using GENSTAT¹⁷⁹.

Results

Between November 1994 and December 1995, 176 women who attended the Menopause Research Unit, Leicester, were randomised to one of the 4 dose groups of trimegestone, 169 were treated and 131 completed the study, while only 129 women were evaluated for endometrial analysis, as 2 women declined to have a biopsy at the end of the study. There were 7 women who did not start treatment after randomisation and were withdrawn from the study as a result of being lost to follow up (3), protocol violation (3) and adverse events (1).

Women randomised into the study had an overall mean (SD) age of 52.2 years (5.2), weight of 64.2 kg (9.5), height of 160.8 cm (6.6) and body mass index of 24.8 kg / cm² (3.4) (Table 6. 1). There were no statistically significant differences between the treatment groups in terms of age, race, height, weight, body mass index and smoking habits. The number of women who completed the study were; 33, 33, 37 and 28 in dose groups 0.05mg, 0.1mg, 0.25mg, and 0.5mg; respectively. Thirty eight women did not complete the study due to bleeding problems (9), mastalgia (6), gastro-intestinal tract symptoms (1), headaches (4), depression (2), pruritus (3), muscular cramps (1), weight gain (1), weight loss (1), GIT cancer (1), breast cancer (1), deep venous thrombosis (2) women's request (4) and lost to follow up (2). There was no statistically significant difference between the number of women who withdrew from each trimegestone dose group.

Six hundred and ten diaries were analysed. All women who completed the study experienced an episode of bleeding in each treatment cycle. The diaries were evaluated for the day of onset, duration and severity of the progestogen associated bleeding and episodes of IMB. Submucous fibroids were identified at baseline hysteroscopy in 4, 7, 5 and 7 women in doses of 0.05mg, 0.1mg, 0.25mg and 0.5mg dose groups respectively (Figure 6. 1). While endometrial polyps were identified in 3, 3, 5 and 2 women assigned to 0.05mg, 0.1mg, 0.25mg and 0.5mg dose groups, respectively. Age, duration of the menopause, previous use of HRT, height, weight, or body mass index did not influence the bleeding pattern among the 4 trimegestone dose groups.

Analysis of the bleeding diaries showed that the episodes of progestogen associated bleeding lasted longer ($P=0.026$) and were heavier ($P=0.002$) in the presence of submucous fibroids (Table 6. 2). This effect was apparent only in the higher 3 doses of trimegestone. The odds of having mean total bleeding score of over 17.75 (the 3rd quartile) was more than 4 times higher in women with submucous fibroids than without (odds ratio 4.54, 95% C.I; 1.45, 14.26).

The IMB occurred more frequently in the presence of submucous fibroids ($P=0.017$) (Figure 6. 2). However, there was no statistically significant association between submucous fibroids and the duration ($P=0.83$) or the severity ($P=0.27$) of the intermenstrual bleeding (data

not shown). There was no statistically significant effect of the endometrial polyps on any of the bleeding parameters studied.

The PAB episodes occurred progressively later in the treatment cycle with the increase in the dose of trimegestone ($P < 0.001$), and the PAB episodes were consistently shorter ($P < 0.001$) and lighter ($P < 0.001$) in the higher doses (Table 6. 2). The episodes of IMB were lighter in the higher dose group ($P=0.035$), but there was no demonstrable dose response effect on the incidence or the duration of the IMB.

Summary of the study

I assessed the effect of endometrial structural abnormalities as diagnosed by outpatient hysteroscopy in 176 postmenopausal women who were given trimegestone-based HRT at baseline. The number of submucous fibroids were 4, 7, 5 & 7 in the doses 0.05mg, 0.1mg, 0.25mg & 0.5mg respectively. There were 9 submucous fibroids grade 1, 10 grade 2 and 10 grade 3.

Submucous fibroids were predictive of heavier and longer PAB, with a higher incidence of IMB, however this effect was second to the effect of the dose of trimegestone.

The presence of endometrial polyps had no effect on the pattern of bleeding.

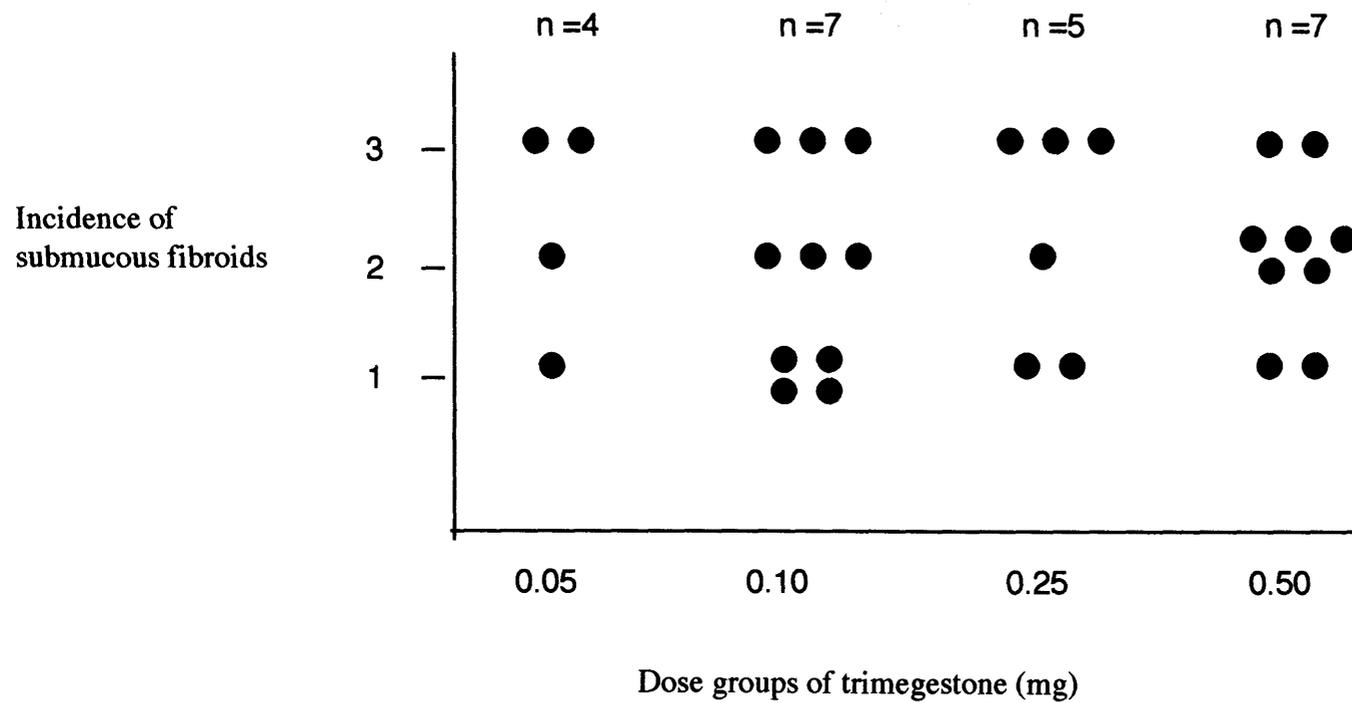


Figure. 6. 1. The incidence of hysteroscopically identified submucous fibroids, and their grades. It can be noted that a woman may have had more than one fibroid. (n = number of women in a particular dose group).

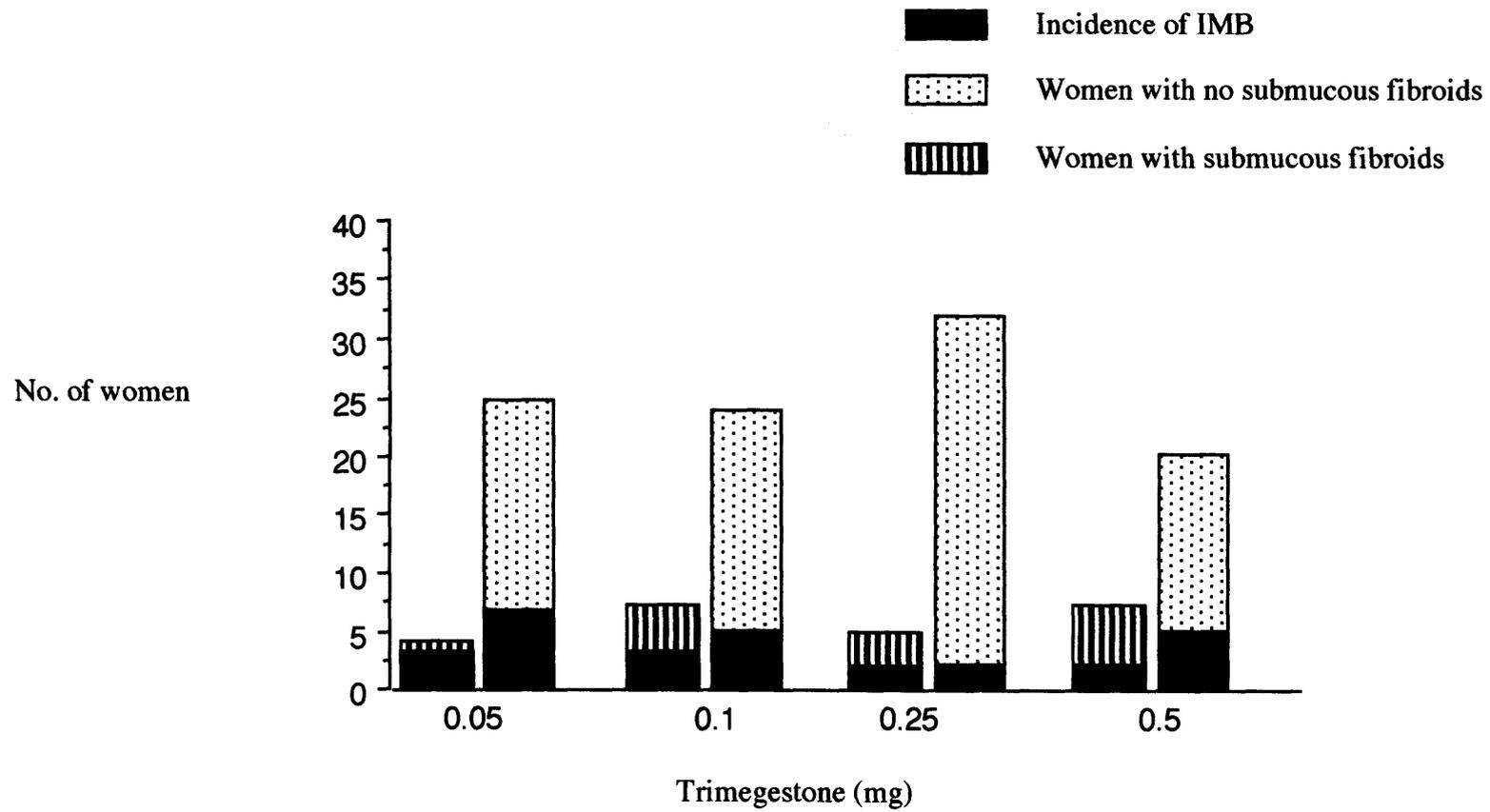


Figure. 6. 2. The incidence of intermenstrual bleeding in women with and without submucous fibroids in the 4 doses of trimegestone.

Table 6.1. Demographic characteristics of the trimegestone-treated women who underwent hysteroscopy at baseline and at the end of the study, and the number of withdrawals in each dose group

	Total	0.05 mg	0.1 mg	0.25 mg	0.5 mg
No. of women treated	169	43	44	44	38
Race					
White	160	40	42	40	38
Black	1	0	0	1	0
Asian	8	3	2	3	0
Age (years), mean (SD)*	52.5 (5.1)	52.6 (5.1)	52.6 (5.3)	53.2 (4.3)	51.5 (5.6)
Weight (kg), mean (SD)*	64.2 (9.3)	64.3 (10.5)	64.1 (9.7)	65.3 (9.3)	62.8 (7.5)
Height (cm), mean (SD)*	160.9 (6.7)	160.6 (7.5)	160.1 (6.9)	161.7 (6.9)	161.1 (5.3)
BMI [®] (Kg/m ²), mean (SD)*	24.8 (4.3)	24.9 (3.6)	25 (3.4)	25 (3.3)	24.2 (2.8)
Smoking					
Smoker	39	10	11	10	8
Given up smoking	25	7	7	4	7
never a smoker	105	26	26	30	23
No. of women withdrawn	38	10	11	7	10
Women completed the study	131	33	33	37	28

(SD)* = Standard Deviation, BMI[®] = Body Mass Index

Table 6. 2. The mean and standard deviation (SD) of the day of onset, duration and severity of the progestogen associated bleeding (PAB) in women with and without submucous fibroids. The SD are across the cycles and includes variations within the women and between women. TBS: Total bleeding score

a. The bleeding pattern in women with submucous fibroids

Dose Groups	No. of women	Cycles	Mean day of Onset (SD)	Mean duration (SD)	MeanTBS (SD)
0.05mg	4	20	28.4 (3.5)	7.0 (2.6)	16.3 (7.0)
0.1mg	7	35	27.6 (2.9)	7.9 (2.7)	19.5 (6.6)
0.25mg	5	26	27.8 (2.3)	6.9 (2.4)	14.5 (6.1)
0.5mg	7	34	30.6 (1.3)	5.8 (1.6)	13.5 (3.9)

b. The bleeding pattern in women without submucous fibroids

Dose Groups	No. of women	Cycles	Mean day of Onset (SD)	Mean duration (SD)	MeanTBS (SD)
0.05mg	25	122	25.5 (3.03)	7.5 (2.7)	16.6 (6.4)
0.1mg	24	124	26.7 (3.29)	6.7 (2.4)	14.6 (5.4)
0.25mg	32	147	29.1 (3.19)	5.8(2.64)	11.7 (5.8)
0.5mg	20	102	30.1 (3.25)	4.5(1.5)	10.7 (4.1)

Chapter Seven
Endometrial Histomorphometry

Introduction

The endometrium of the natural cycle is associated with morphological changes and ends, in the absence of implantation, in endometrial shedding and menstruation. Several investigators have tried to define these endometrial changes as early, mid and late stages of the proliferative or secretory development. However, the histological definition of the phase of endometrial development is based on the whole picture and not on one single parameter since in most tissue sections a greater or lesser extent of mixed structural changes occur. Therefore, when ascribing a particular phase of endometrial development, histopathologists label the tissue, in the presence of mixed structural changes, according to the most advanced feature. In addition, there are difficulties in dating the endometrium in the proliferative phase as early, mid or late proliferative. Not only due to the overlap in the histological characteristics, but also the inter-observer variation between pathologists. In an interesting study, the incidence of complex hyperplasia reported by 3 pathologist blinded to the clinical details and the findings of their colleagues ranged from 1.1% to 5.1%⁵⁴.

Regional variations in endometrial responses to sex steroids have been recognised such that endometrial tissues from the uterine fundus give more reliable information than the isthmus, which may add a further complexity in the histological analysis of endometrial curetting samples. Moreover, the depth of the endometrial sample examined is

equally critical, for example, biopsies that include only the basalis but not the functionalis are not datable ⁵².

Exogenous sex steroids result in withdrawal bleeding and induce changes in all components of the endometrium, which are different from those under the influence of the natural ovarian cycle ^{53,72 51 186 187}. ¹⁸⁸. The effects of contraceptive steroids on the endometrium depend on the type, dose and duration of the steroid administered.

Oral NET given as contraception in 300µg/day, for 3 months, reduces significantly the number and diameter of endometrial glands ¹⁸⁷.

Administered in 50µg/day through a vaginal ring, NET, does not affect the size or number of the glands per unit area, but when the dose is increased to 200µg/day, it significantly reduces the size of the individual gland at 6 weeks, and reduces the number of these glands if given for 10 weeks ¹⁸⁷.

LNG as intra vaginal ring in a dose of 10µg/day had no effect on the endometrial glands, while in 20µg/day for 6 weeks, it was associated with a reduction in glandular diameter, and a reduction in glandular size when the dose was increased to 25µg/day for 6-10 weeks of treatment ¹⁸⁷.

Prolonged exposure to LNG-releasing vaginal device was associated with significant decrease in glandular number and diameter and an increase in glandular and stromal mitoses during the treatment period ¹⁸⁹.

Ludwig (1982)¹⁰⁰ in his study of the effect of different progestogens for a duration between 2 and 38 months found a remarkable increase in stromal oedema when large doses of progestogens were administered for a short period of time. There were lower number of endometrial glands, which has an atrophic epithelium. Basal vacuolation disappeared after few months of treatment, secretory and cellular signs disappear after 2 months of treatment. Foci of pseudodecidualisation were noticed with focal infiltration of round nuclear cells.

External sex steroids modify the endometrial vascularity; Norplant use is associated with increase microvascular density compared to the control ¹⁹⁰, while oral MPA or NET for 5-6 months was associated with reduced vascular density and an increased number of dilated venules ¹⁹¹. Similar finding was reported using LNG-releasing IUD ¹⁹⁰.

Hendrickson & Kempson (1980) described briefly the general changes associated with oestrogen and progestogen administration: there was non-uniformity in glandular development and loss in endometrial volume manifested by underdeveloped non-coiled glands, which contained no secretion or subnuclear vacuolation, while the stroma showed premature decidualisation and patchy oedema. The spiral arteries were underdeveloped ¹⁹².

Progestin effect can last for months after cessation of progestin use. The pattern of changes induced with progestogen treatment are; decidual (pregnancy like changes), secretory and atrophic changes, although, usually there is an overlap between these various patterns⁵⁵.

Arrested or aborted secretions have been described following progestogen administration for 3 months; where proliferation of the endometrial glands is arrested. The glands are sparse and lined by atrophic epithelium. If treatment continuous for many months, arrested secretion may change to fibrous atrophy. This usually develops in combination treatment with predominance of progestogen⁵³.

There is paucity in the literature describing the histological changes associated with hormone replacement therapy in postmenopausal women. Casanas et al. (1996) studied the morphometric characteristics associated with oral oestrogen and cyclically administered progesterone as vaginal gel and found an increase in glandular coiling in response to this regimen which mimicked the glandular changes in the luteal phase¹⁸⁸. Habiba et al. (1998) assessed the histological changes associated with cyclical sequential combined orally administered oestrogen and NET⁵¹.

Most of these histomorphometric studies suffered either from small sample size or unclear method of assessment, and none address the influence of each histological parameter on the overall histological picture.

Aims

I evaluated detailed histological features relevant to endometrial developments in women treated with trimegestone-based HRT and during the natural cycle using image analysis techniques and linear discriminant analysis to examine :

- a. Specific morphological changes induced in the endometrium by the sequentially administered trimegestone in a dose ranging study.
- b. To compare the findings to those measured in 5 definable stages of endometrial development of the natural cycle.
- c. The potential existence of specific structural changes in the endometrium under this trimegestone-based HRT regimen, which may be associated with an observed bleeding pattern.

Women and method

The study population of 176 women are described in page 51-52.

Endometrial biopsies were obtained on day 24 of the last treatment cycle. The endometrial samples were fixed immediately in 10% formal-saline embedded in paraffin and 4 µm sections were stained with haematoxyline and eosin (H&E) for histological assessment (standard protocol at histopathology department, Leicester Royal Infirmary).

Treatment

Women were randomised to receive one of 4 doses of trimegestone

Page 90.

The control

The characteristics of the control samples (n=37) are described in page 53. The histological diagnoses, were proliferative (n= 8), early luteal (n= 8), mid luteal (n=5), late luteal (n= 8) and menstrual (n= 8).

Assessment of the endometrial sections

Fifteen randomly selected fields ¹⁶⁸ per slide (x200) were captured using image analysis programme to assess 13 variables (page 63). Glands: total glandular area, average glandular diameter, number of complete and incomplete glands per field, glandular epithelial height in the glands (columnar or cuboidal), regularity of the glandular epithelial surface (smooth or irregular), percent of gland containing luminal secretions and subnuclear vacuoles, and number of invaginations (telescoping) in the field. Vascularity was examined for total vascular space area, average vascular space diameter, and number of vascular spaces per field. To assess stromal cellular density, the nuclei were counted in fifteen randomly selected fields ¹⁶⁸ per slide (x1000) under oil immersion. All fields examined were restricted to the functionalis layer.

Images were captured using Axioplan microscope (Carl Zeiss, Herts UK), and a colour video camera (Sony CCD/RGB). The data presented are per unit area and the corresponding areas of an image captured with x200 and x1000 were 0.121mm² & 0.0046 mm² respectively using a measurement graticule. The areas measured or cells counted were

evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, UK).

Statistics

For each specimen 15 fields were taken, and the measurements for each field were averaged to give a mean score and standard deviation for that specimen. Kruskal-Wallis nonparametric one way ANOVA and Wilcoxon's rank sum test (Mann-Whitney test) were used to compare individual measurements between the 4 dose groups and between phases of the natural cycle. Wilcoxon's rank sum test was used to compare the histological parameters between women who bled on the day of the biopsy and those who had not bled by then. The average scores for each specimen on 13 variables were then analysed by linear discriminant analysis ¹⁹³ to look for differences between the four treated groups and the five phases of the natural cycle.

Results

In the MRU, Leicester, 176 women were randomised to one of the 4 dose groups and 131 completed the study, their demographic data are as described in page 126. Two women declined to have a biopsy at the end of the study, and therefore the total number of endometrial samples obtained at the end of the study was 129. Endometrial biopsies of women who bled before the day of the biopsy were excluded for consistency, particularly as the endometrium would start the healing-proliferation phase postmenstrum (n = 12). Twenty four other samples were too scanty for

meaningful analysis. Ninety three endometrial biopsies were evaluable for morphometric assessment, (n = 20, 20, 28, and 25 in the 0.05mg, 0.1mg, 0.25mg, 0.5mg trimegestone dose groups, respectively, Figure 7.1).

a. The endometrium of the natural cycle (Figure 7.2 a-e shows the phases of the natural cycle)

The mean total glandular area (Figure 7. 3a) was higher in the luteal phases compared to the proliferative phase (early luteal; $P = 0.0002$, mid luteal; $P = 0.0007$, late luteal; $P = 0.003$). The average glandular diameter (Figure 7. 3b), was higher in the mid luteal and menstrual phases compared to the proliferative phase, but these differences were not statistically significant. The height of the glandular surface epithelium was lowest in the mid luteal ($P < 0.004$), but started to increase gradually in the late luteal and menstrual phases. The average number of the endometrial glands per unit area did not differ significantly in the 5 phases of the natural cycle (Figure 7. 3c). Endometrial glandular epithelial vacuoles did show variation across the phases ($P = 0.0002$), and was significantly higher in the early luteal phase compared to any one of the other phases (all $P < 0.004$). There was some evidence of a difference in the glandular secretions between the phases of the natural cycle ($P = 0.03$, Figure 7. 3d) due to a slightly higher values in the mid and late luteal phases.

The mean total vascular space area increased gradually as the luteal phase advanced and was highest in the menstrual phase ($P < 0.05$; Figure 7. 3e). The average vascular space diameter varied between the

phases of the natural cycle (Figure 7. 3f). There was no suggestion of a difference in the mean number of vascular spaces per unit area in the 5 phases of the natural cycle (Figure 7. 3g). There was no difference in the stromal cellularity between the different phases of the natural cycle (Figure 7. 3h).

b. The trimegestone-treated endometrium

The endometrium of the HRT cycle, irrespective of the dose, had significantly smaller mean total glandular area compared to the luteal phase ($P < 0.0001$; Figure 7. 3a), and slightly smaller average glandular diameter than the luteal phase (Figure 7. 3b). There was no difference in the mean number of glands per unit area when compared to the natural cycle (Figure 7. 3c). The height of the glandular epithelium was significantly lower compared to the phases of the natural cycle ($P < 0.0004$). Glandular telescoping (Figure 7.2f), was more prevalent in the 0.1 and 0.25 mg doses of trimegestone treated endometrium, but were generally lower than the early and late luteal phases, however, it is difficult to draw any conclusion, since there was no telescoping in the proliferative or mid luteal phases. The higher dose of trimegestone was associated with significantly lower glandular secretions compared to the lower doses, or to the phases of the natural cycle ($P < 0.005$, Figure 7. 3d and $P < 0.04$, Figure 7.2g, respectively).

Irrespective of the dose, the mean total vascular space area

(Figure 7. 3e) were smaller than those in the late luteal and menstrual phases ($P < 0.0001$). Similarly the average vascular space diameter (Figure 7. 3f) was smaller than in the natural cycle ($P < 0.001$). However, there was no difference in the mean number of the vascular spaces in the HRT cycle compared to the natural cycle (Figure 7.3g). Stromal cell count did not differ between the 4 dose groups or in comparison to the natural cycle (Figure 7. 3h).

There was no correlation between the histological parameters studied and the bleeding patterns in women who bled by the time of the biopsy or those who did not. Telescoping was more prevalent, however, in women who had not bled by the time of the biopsy compared to those who had already bled on that day ($P < 0.002$).

Linear Discriminant Analysis

Table 7.1 shows the mean scores for the 13 histological variables described above in the four treated groups and the five phases of the normal cycle. The within group standard deviation and correlations between the endometrial parameters are shown in table 7. 2. The average number of vascular spaces, the mean total vascular area, and average vascular space diameter are highly correlated. High correlation was also found between the mean total glandular area and average glandular diameter. To highlight the differences between the groups linear discriminant analysis was performed. The measurements were standardised by subtracting the overall mean from the individual

observation value and dividing by the pooled within group standard deviations so that the effects of the scale of measurement are removed and the relative contributions of the different measurements are more clearly seen.

The linear discriminant functions (Table 7. 3) produce weighted combinations of the 13 measurements which best separate the nine groups (4 doses of trimegestone, and 5 phases of the natural cycle). These functions are ordered so that the first linear discriminant function shows most discrimination, followed by the second, third, etc. The signs and sizes of the standardised weights show the importance of each variable to the final score. Thus mean total glandular area contribute highly to the score of linear discriminant function 1, while second linear discriminant function is associated with higher percentage of glands with vacuolation or telescoping, but lower mean vascular space area and lower percentage of glands with columnar epithelial lining.

Table 7. 4 shows the mean scores for women in the nine groups for each of the first four linear discriminant functions. The average scores for each group and scores for the individual woman on the first two linear discriminant functions are plotted in figure 7.4a.

On the basis of these measurements, the trimegestone treated endometria appear to be very similar, regardless of the dose. The first linear discriminant function separates the luteal phases from the

trimegestone treated group and the second linear discriminant function separates the proliferative, menstrual, and mid luteal phases from the trimegestone treated groups. Therefore, the averages for the trimegestone treated groups can be distinguished from the averages of each of the phases of the natural cycle, although the variation between the tissue specimens within these groups is substantial and there is considerable overlap.

If this first plot (Figure 7. 4a) is rotated anti-clockwise through 30° the scales, the first rotated linear discriminant functions will then distinguish between the treated and control groups and the second rotated linear discriminant functions distinguish between the phases of the natural cycle (Figure 7. 4b). The standardised weights for defining the rotated linear discriminant functions are shown in Table 7. 5.

The rotated linear discriminant functions depend most critically on just a few variables. If only the variables with standardised coefficients greater than 0.4 are used, then discriminant functions are obtained that capture most of the discriminatory information in this data set. The resulting plot is shown as Figure 7. 4c. Most of the discriminatory information is retained although the proliferative phase is now less well distinguished from the treated groups.

Using the original raw data to reconstruct figure 7.4c, the following equations can be used to assign a value for the first and second linear

discriminant functions, where it can be compared to the different phases of the natural cycle or to the trimegestone-based HRT regimen

a. 0.139 (mean % total glandular area) + 0.797 (mean % total vascular space area)

b. 0.127 (mean % total glandular area) - 0.909 (mean % total vascular space area) - 0.029 (average glandular diameter) + 0.061 (average vascular space diameter) + 0.019 (% glands with vacuolation).

Summary of the study

In this study, I assessed 13 histological parameters in the endometrium of the natural cycle and endometrial samples following treatment with trimegestone-based HRT, using image analysis programme.

The total glandular area increased in the luteal phase, but there was no difference in their number in different phases of the natural cycle, while glandular epithelial lining became progressively taller as the phases of the cycle advanced. There were more vacuoles in the early luteal phase, while glandular secretion was higher in mid and late luteal phases. Total vascular space area increased as the phases of the natural cycle advanced, but there was no statistically significant difference in the mean number of the blood vessels.

In the trimegestone-treated endometria, the total glandular area and diameter were smaller than in the endometrium of the natural cycle, while there was no difference in the glandular number per unit area. The total vascular area and diameter in the trimegestone-treated samples were smaller than in the endometrium of the natural cycle, while there was no difference in the mean number of blood vessel.

The only difference between the 4 doses was a lower glandular secretion in the 0.5 mg dose. More telescoping was observed in endometria of women who had bled at the time of the biopsy compared to women who did not bleed by then.

Linear discriminant analysis was used to look at standardised weighted values of the histological parameters that best distinguish the HRT-treated endometrium from that of the natural cycle. The trimegestone-treated endometria were very similar. The first linear discriminant function (LDF) discriminate between the trimegestone-treated endometria and the luteal phases, while the second LDF separate the trimegestone-treated endometria from the proliferative, menstrual and mid luteal phases (Figure 7.3a). If this plot is rotated, then the first rotated LDF will distinguish between the treated group and the control, while the second rotated LDF distinguish between the phases of the natural cycle.

Weighted values were used to construct two equations, which aid to assign a position of evaluated parameters of an endometrial samples on the linear discriminant plot. This may serve to compare the effect of a particular HRT treatment on tissue morphometry to those observed during the phases of the natural cycle.

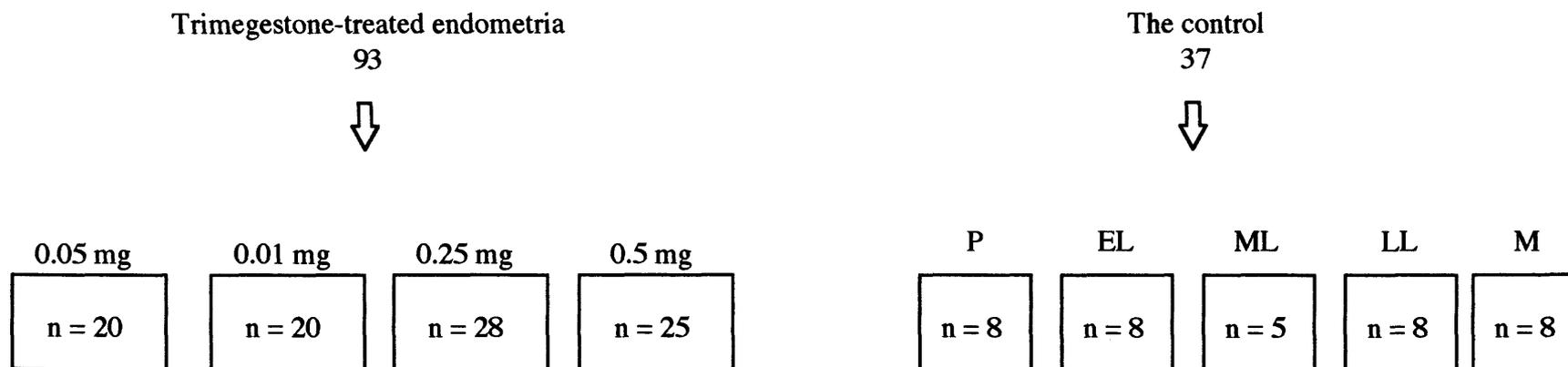
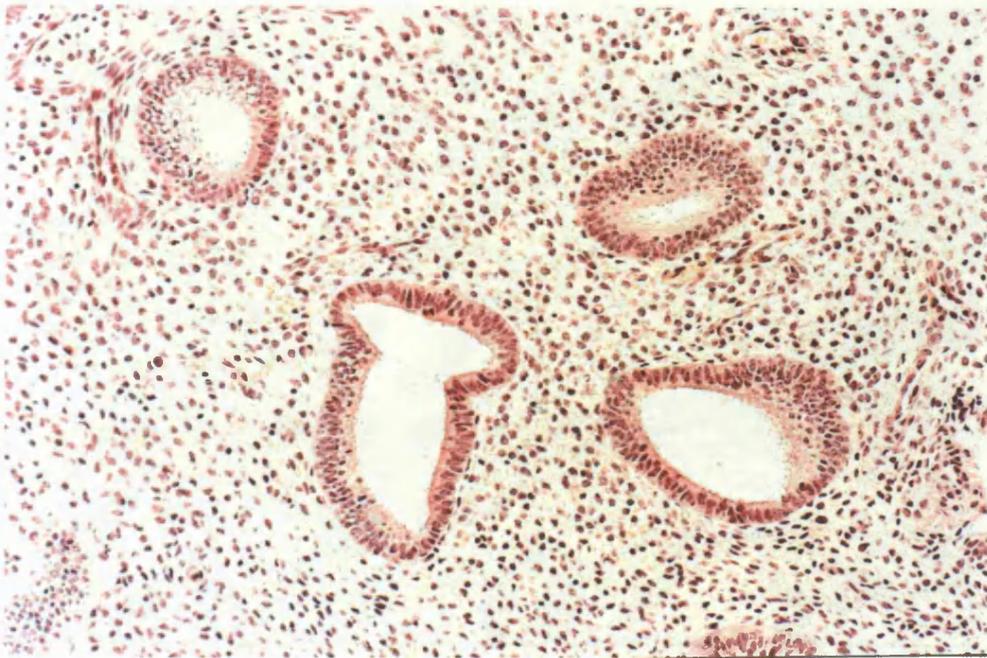
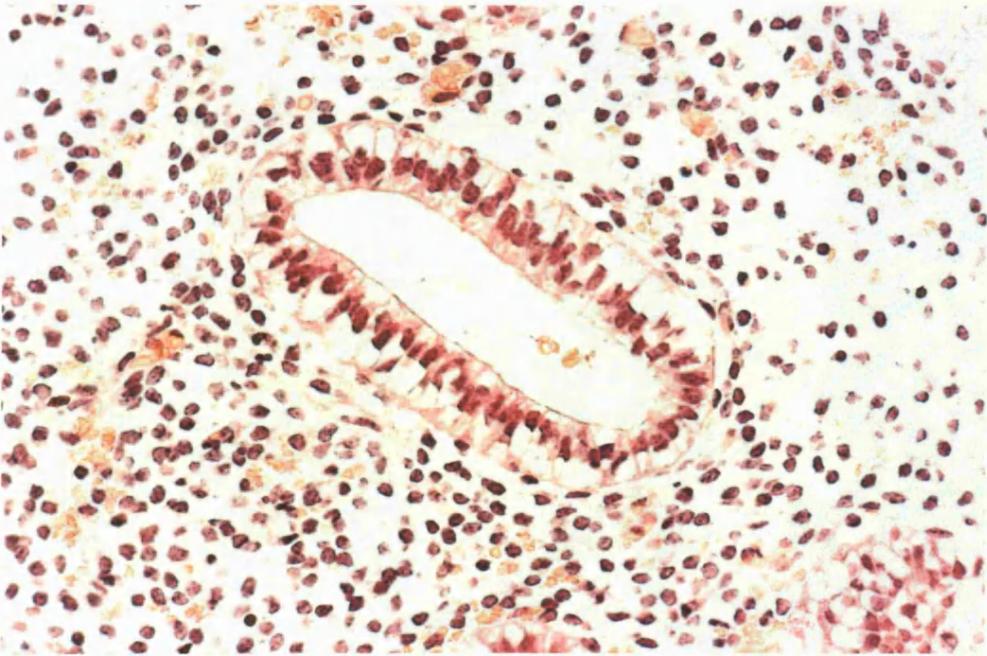


Figure 7.1. Number of specimens in the Histomorphometry study. Total number of specimens were 93, as women who bled before the day of the biopsy were excluded (n = 12), and some specimen were too scanty for evaluation (n = 24), P = proliferative, EL = early luteal, ML = mid luteal, LL = late luteal & M = menstrual.

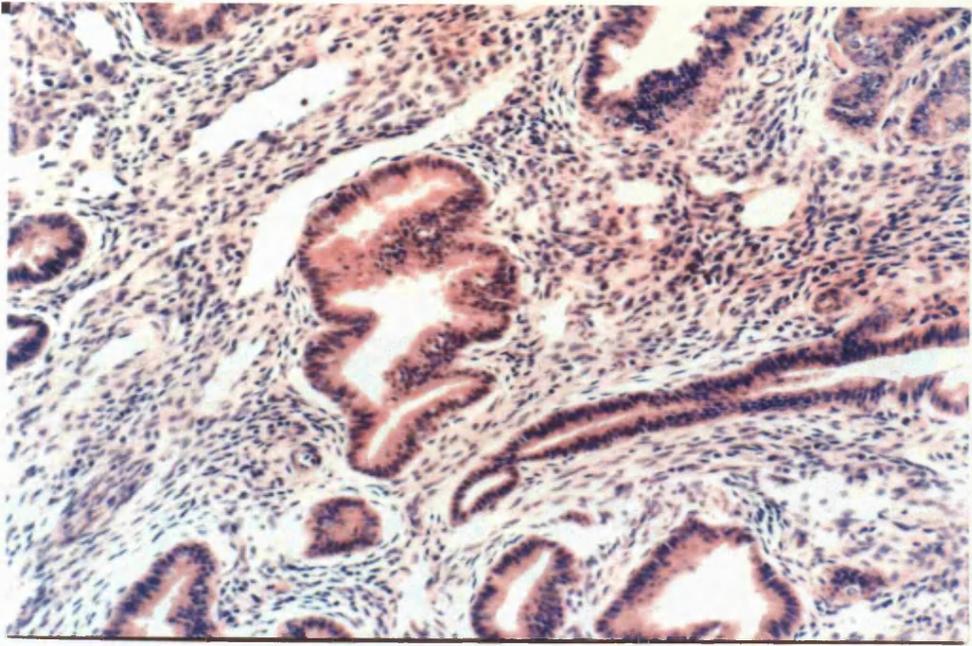


7.2a

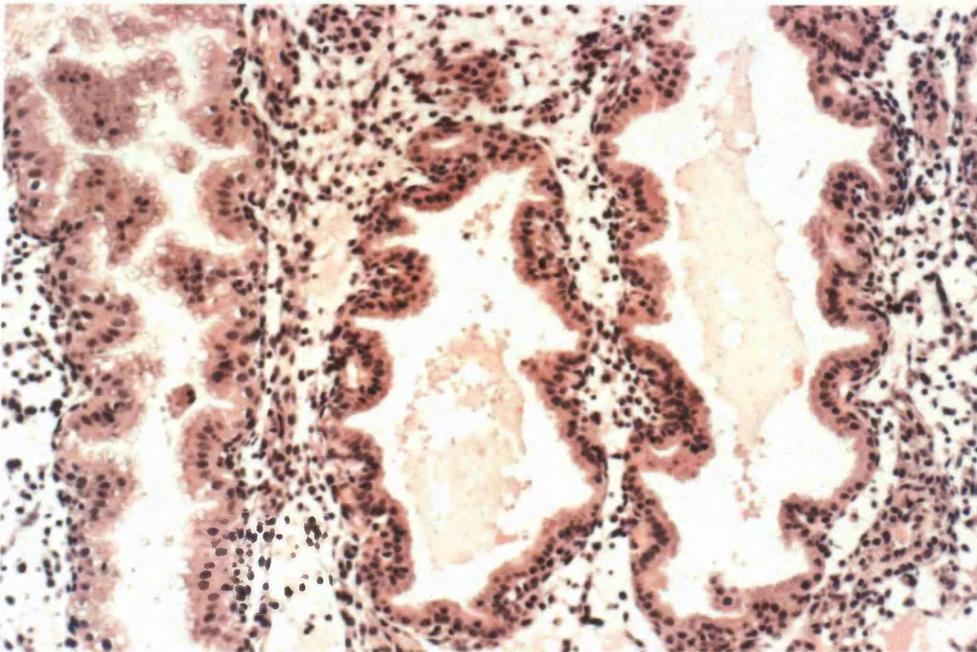


7.2b

Figure 7. 2a & 2b. H&E of the proliferative phase, original magnification x 200, 1cm = 100 μ m (2a), and early luteal phases showing glandular subnuclear vacuoles, original magnification x 400, (2b), 1cm = 50 μ m.

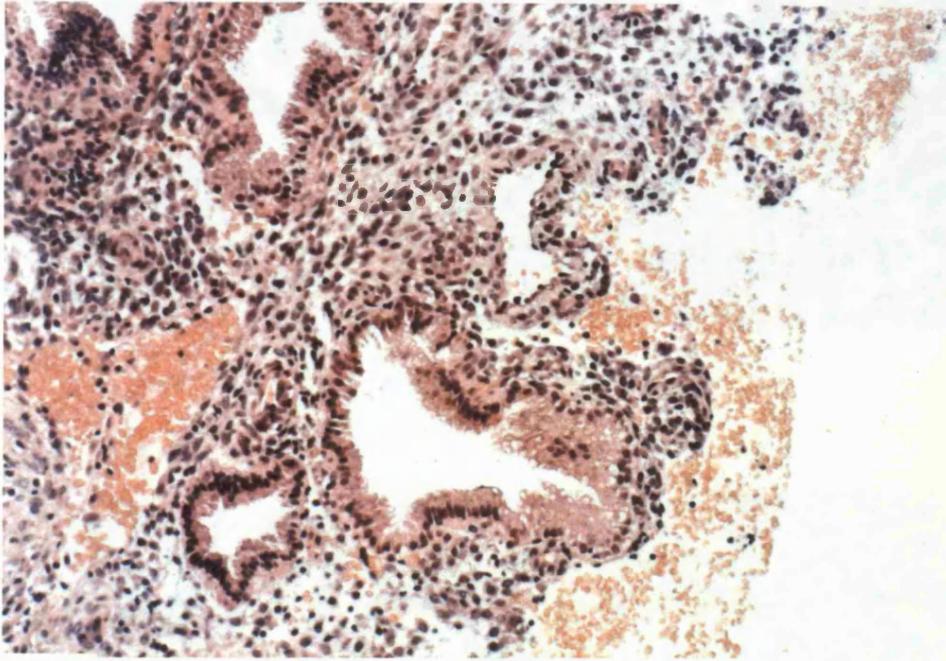


7.2c



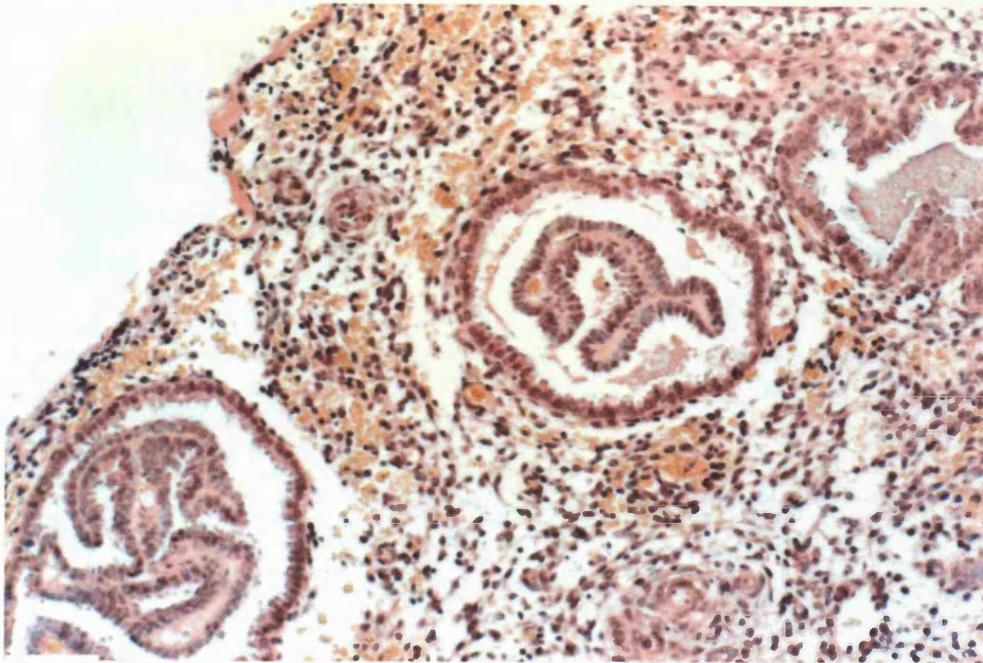
7.2d

Figure 7. 2c & 2d. H&E of the mid luteal phase, original magnification x 200, 1cm = 100 μ m. (2c) and late luteal phases showing glandular tortuosity and secretions, original magnification x 200, 1cm = 100 μ m. (2d).

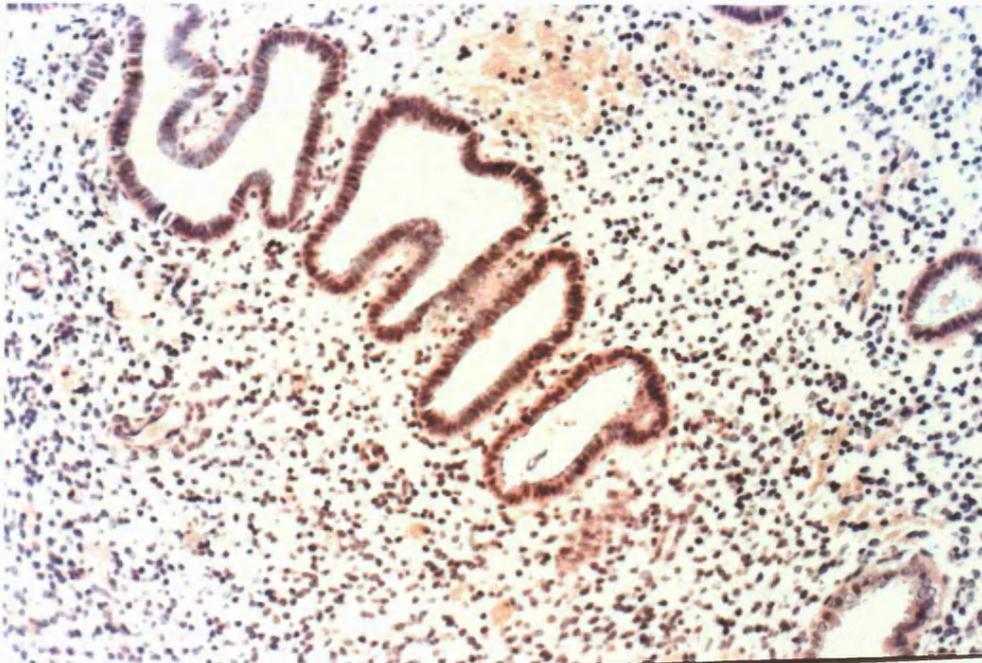


7.2e

Figure 7. 2e. H&E of the menstrual phase showing tissue disintegrations, original magnification x 200, 1cm = 100 μ m.



7.2f



7.2g

Figure 7. 2f & 2g. H&E of the endometrium of the trimegestone-based HRT showing glandular telescoping, original magnification x 200, 1cm = 100 μ m (2f), aborted secretion on the 0.5 mg dose, original magnification x 200, 1cm = 100 μ m (2g).

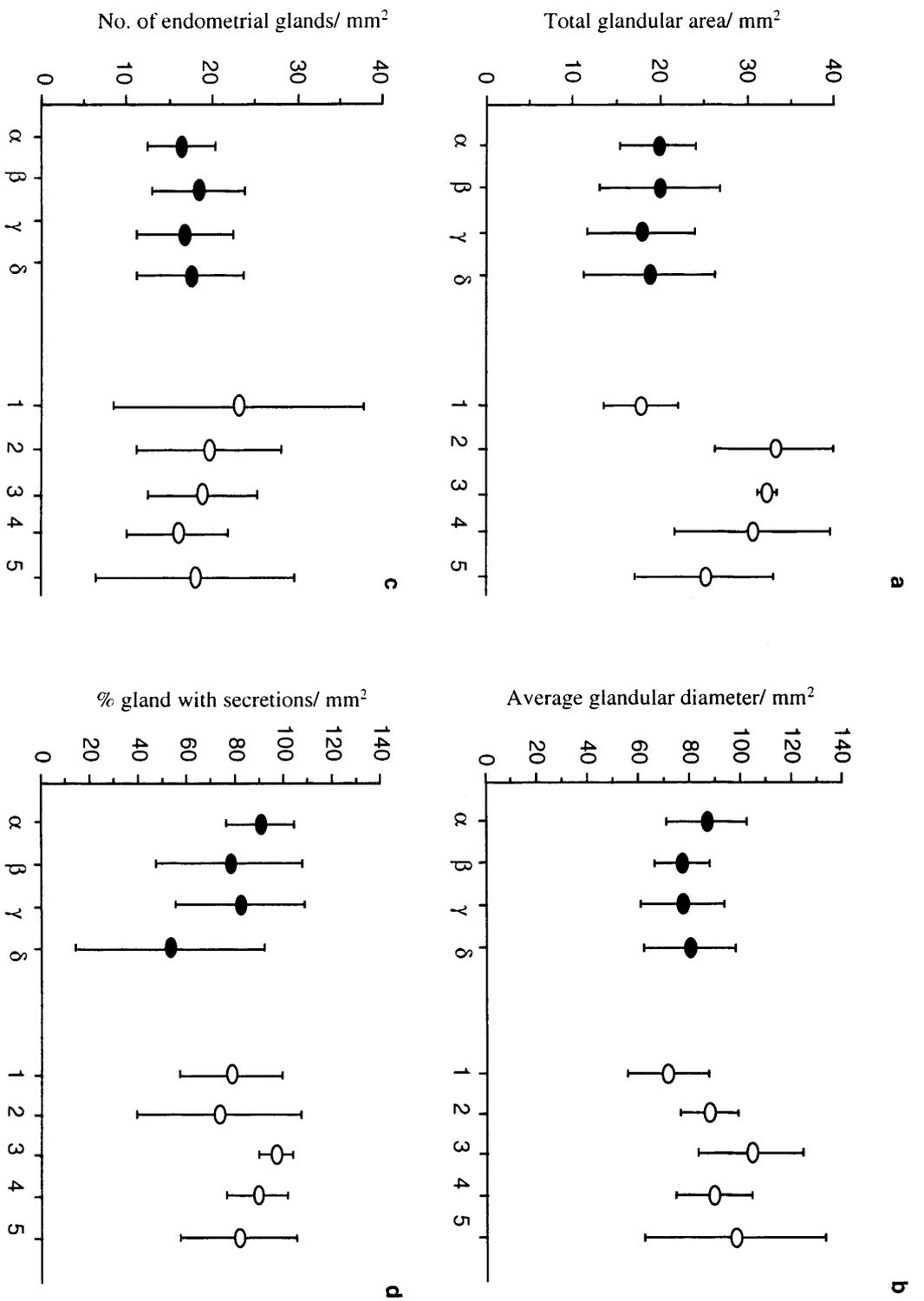


Figure 7. 3a-d (see legend on page 158)

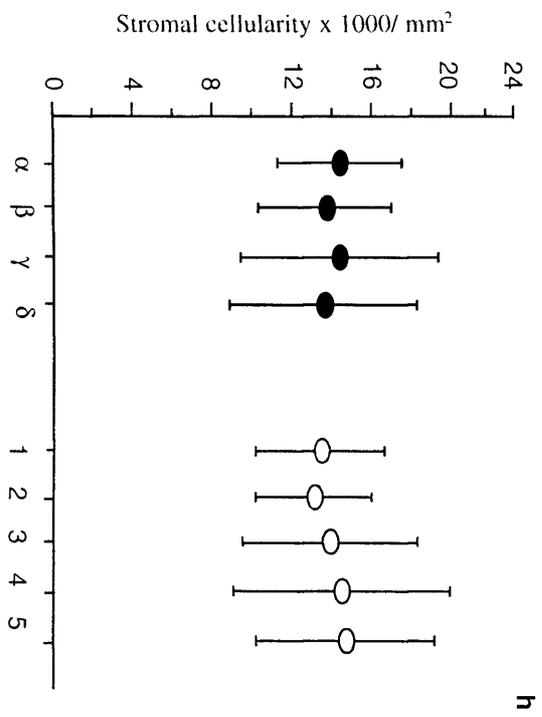
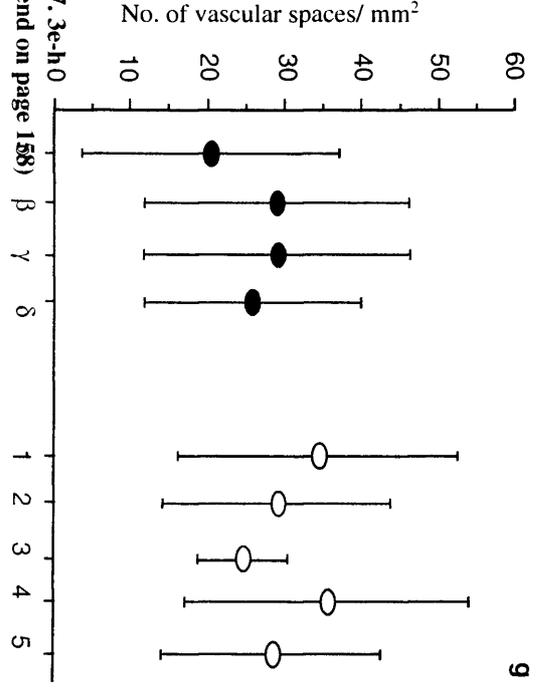
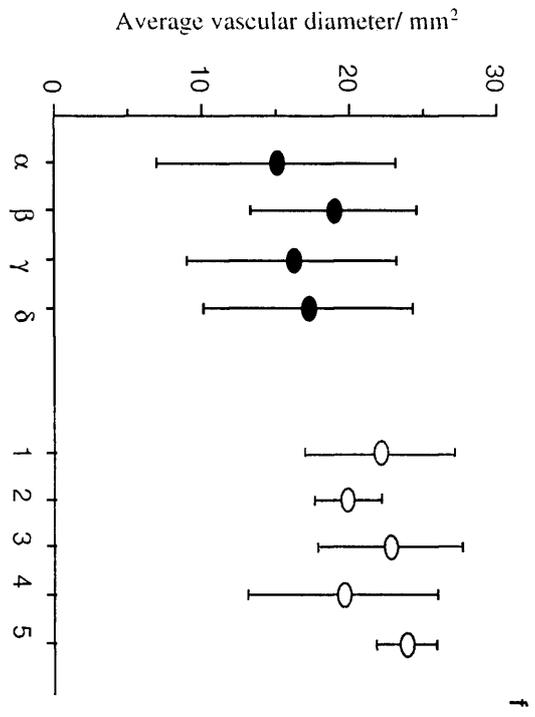
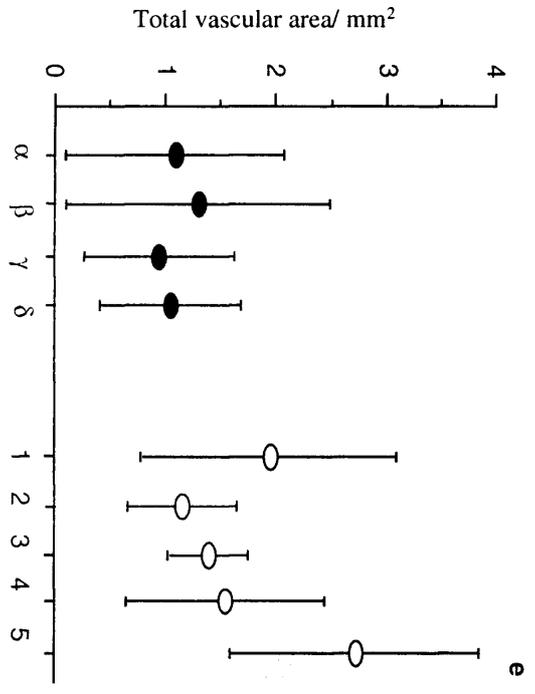


Figure 7. 3e-h0
(see legend on page 158)

Figure Legends

Figure 7.3 (a - h): Mean and standard deviation of areas and cell counts examined in endometrial samples obtained from the 4 dose groups of trimegestone (α , β , γ , δ corresponding to 0.05, 0.1, 0.25, and 0.5mg, respectively, filled circles), and the endometrium from the five phases of the natural cycle (1, 2, 3, 4, 5, corresponding to proliferative, early secretory, mid secretory, late secretory and menstrual, respectively, empty circles). **a.** Total glandular area, **b.** Average glandular diameter, **c.** Number of complete glands, **d.** % of glands with secretions, **e.** total vascular space area, **f.** average vascular space diameter, **g.** number of vascular space area, and, **h.** stromal cellularity.

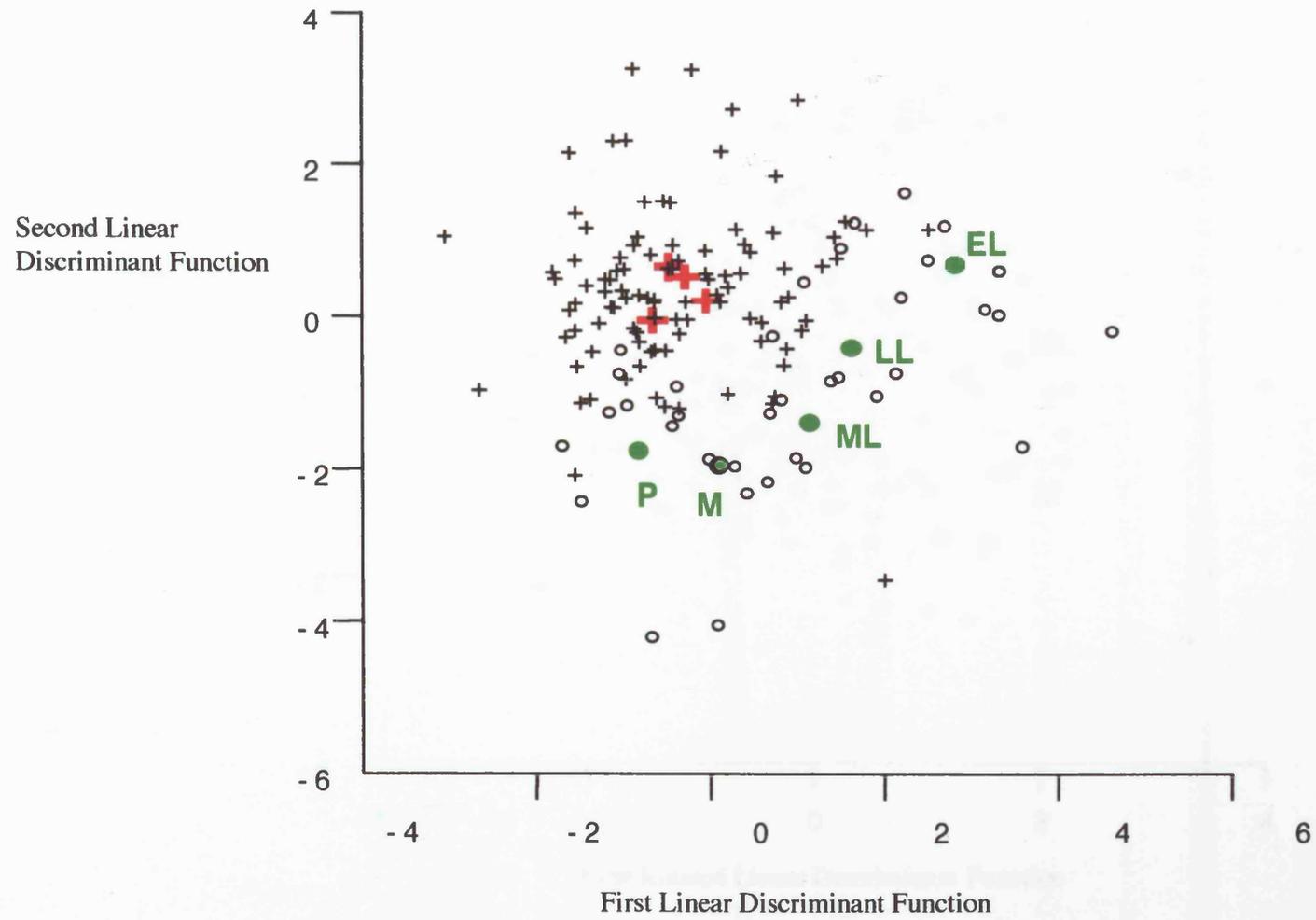


Figure 7.4a

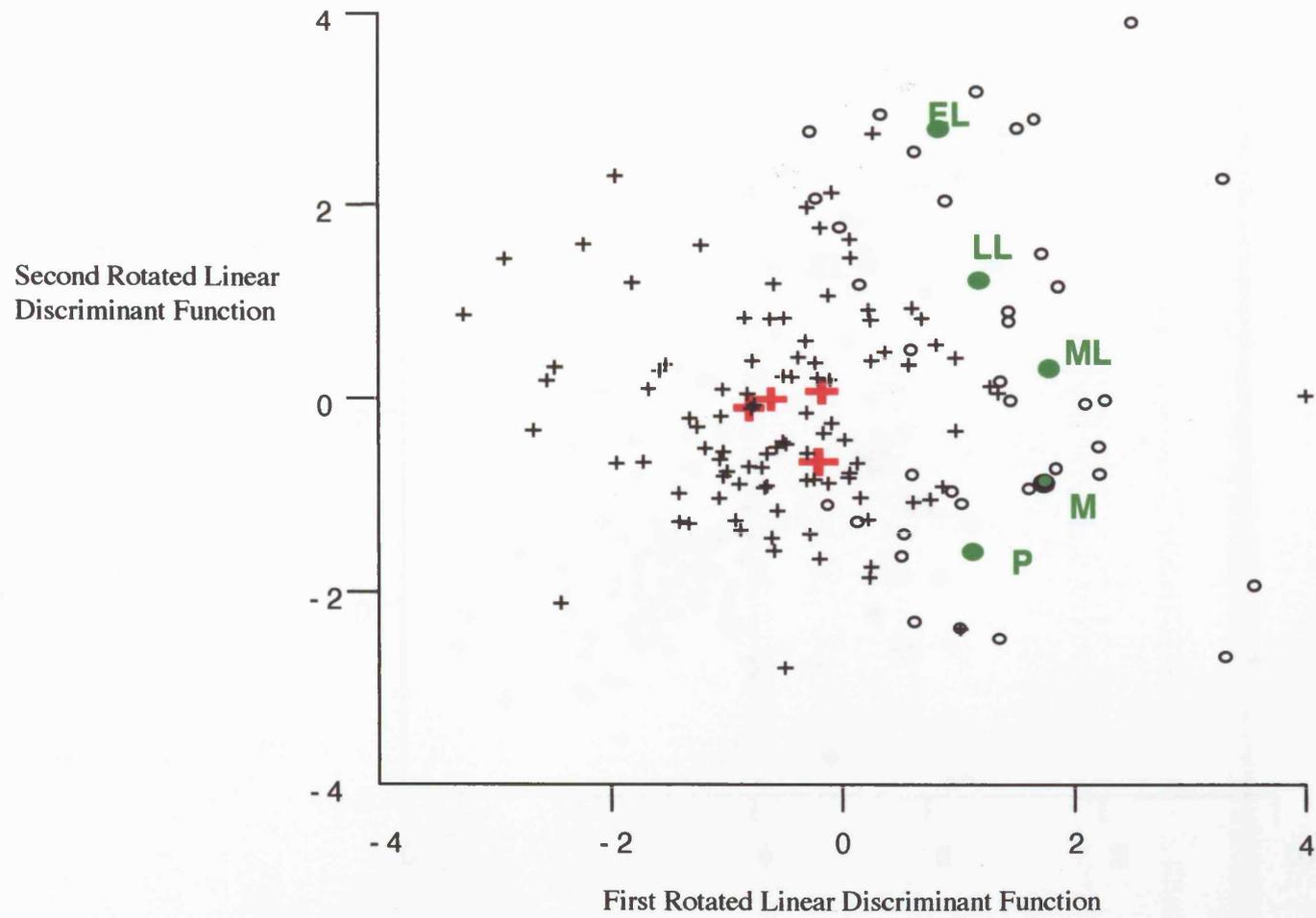


Figure 7.4b

Second Simplified
Linear Discriminant
Function

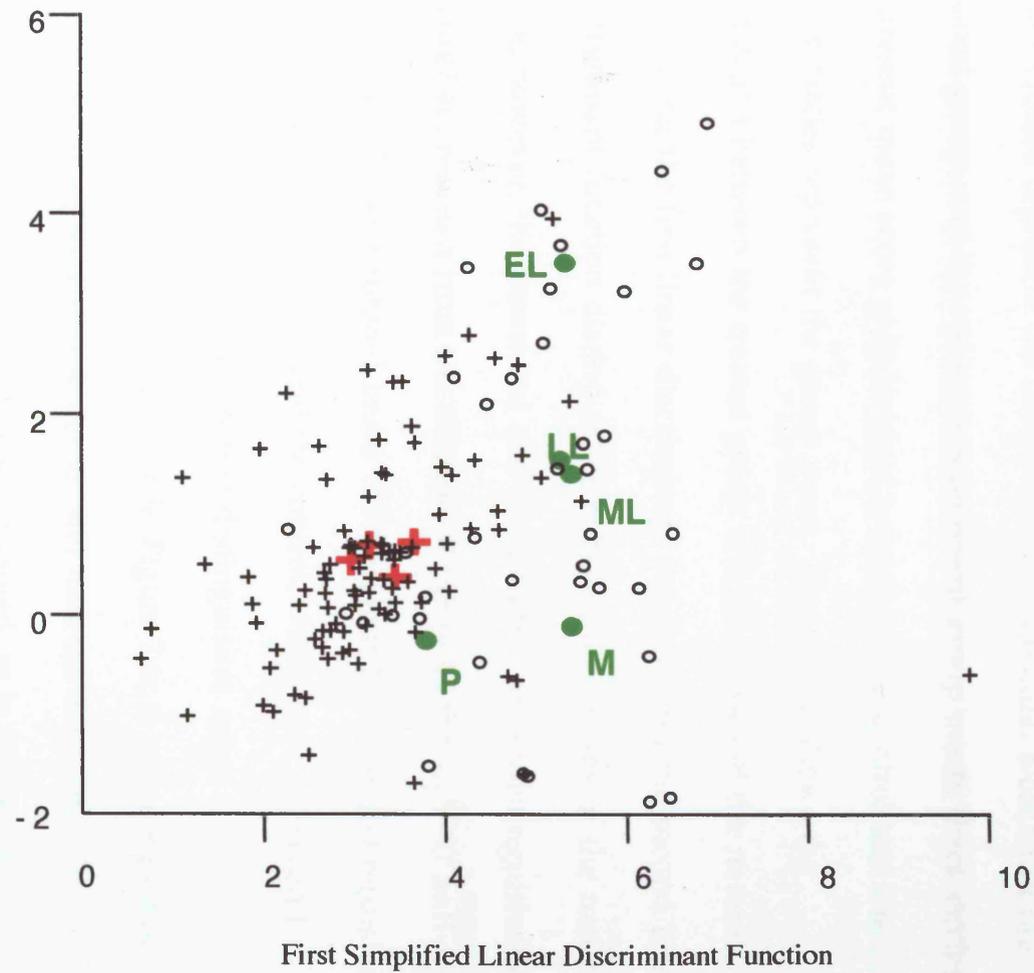


Figure 7.4c

Figure 7.4: The relationship between linear discriminant analysis scores of the histological parameters in the trimegestone treated endometrium and the 5 phases of the natural cycle (P= proliferative, EL= early luteal, ML= mid luteal, LL= late luteal & M= menstrual). Black crosses represent the mean score of individual woman in the treated group, and the red crosses represent group mean. Black circles represent mean score of individual women in the control and the green circles represent the group mean. Figure 7.4a shows the distinction between the treated group and the phases of the natural cycle along the first linear discriminant function, and the second linear discriminant function distinguishes between the phases of the natural cycle, however, the menstrual and proliferative are indistinguishable. Figure 7.4b, resulted from the rotation of figure 7.4a through 60° anti-clockwise. The first rotated linear discriminant function distinguishes between the treated group and the control, while, now the second rotated linear discriminant function distinguishes more clearly between all phases of the natural cycle. Figure 7.4c is a repeat plot of figure 7.4a using weighted scores greater than 0.4 only. The discrimination between the groups is retained, as in figure 7.4a, however the proliferative phase is now less distinguished from the treated group.

Table 7.1 : Mean values for individual histological parameters studied in the endometrium treated with sequential trimegestone and the natural cycle. Data are per mm² endometrial tissue.

Histological parameters	Trimegestone dose groups					Endometrium of the natural cycle ^a					K-W ^b
	0.05	0.1	0.25	0.5	P	EL	ML	LL	M		
	n = 20	20	28	25	8	8	5	8	8		
% glandular area Mean (SD)	18.3 (4.4)	18.4 (6.9)	16.1 (6.2)	17.1 (7.5)	16.2 (4.2)	31.6 (7)	30.9 (0.6)	29 (8.9)	23.2 (7.9)	P< 0.0001.	
% vascular space area	1.0 (0.9)	1.3 (1.1)	0.9 (0.6)	1.0 (0.6)	1.9 (1.1)	1.1 (0.4)	1.3 (0.3)	1.5 (0.9)	2.7 (1.1)	P< 0.003	
Average glandular diameter	86.4 (15.8)	77.1 (10.6)	76.2 (16.3)	79.5 (17.7)	70.4 (15.7)	86.4 (11)	104.5 (19.6)	89 (15)	97.8 (35.6)	P< 0.05	
Average vascular space diameter	15.0 (8.1)	18.9 (5.6)	16.1 (7.1)	17.1 (7.1)	22 (5)	19.8 (2.3)	22.4 (5.1)	19.5 (6.4)	23.9 (2)	P< 0.02	
Stromal cell count ^c	66.2 (14.6)	63.0 (15.2)	65.3 (22.1)	63.1 (21.8)	61.7 (14.9)	60.1 (13.2)	63.5 (20.1)	66.2 (25)	67.3 (20.5)	NS	
No. of complete glands ^d	1.9 (0.4)	2.1 (0.6)	2.0 (0.6)	2.1 (0.7)	2.8 (1.7)	2.3 (1)	2.2 (0.7)	1.9 (0.7)	2.1 (.4)	NS	
No. of 'half' glands ^d	1.2 (0.5)	1.3 (0.5)	1.1 (0.4)	1.1 (0.5)	1.3 (0.3)	2.4 (0.7)	1.2 (1)	2.1 (0.7)	1.2 (0.2)	P< 0.0002	
% gland with columnar lining	65.2 (26.9)	59.9 (22.5)	61.4 (28.0)	69.7 (28.2)	94.8 (11.9)	99.6 (0.7)	56 (44.4)	73.3 (30.5)	70.5 (30.6)	P< 0.0003	
No. of vascular spaces ^d	2.4 (2.0)	3.4 (2)	3.2 (2)	3.1 (1.7)	4.1 (2.2)	3.4 (1.7)	3 (0.7)	4.2 (2.2)	3.4 (1.7)	NS	
% gland with vacuoles	30.1 (39.7)	32.3 (32.3)	32.7 (35.3)	35.5 (41.4)	6.0 (8.7)	95.9 (7.3)	20.2 (32.7)	33.8 (36.1)	40.6 (40.3)	P< 0.002	
% gland with secretions	90.7 (14.3)	78.1 (30.2)	82.3 (26.4)	53.5 (38.9)	77.9 (21.2)	74.1 (34.3)	96.4 (6.9)	88.2 (12.3)	80.4 (23.9)	P< 0.04	
% gland with telescoping	0.04 (0)	0.13 (0.3)	0.11 (0.2)	0.06 (0.1)	0 (0)	0.18 (0.1)	0 (0)	0.18 (0.3)	0.06 (0)	P< 0.03	
% gland with Smooth surface	11.7 (14.8)	24.7 (23.7)	23.0 (22.8)	24.8 (22.7)	21.9 (23)	12.8 (7.4)	10.8 (8.7)	9.7 (13.4)	27.3 (34.6)	NS	

^a P = proliferative, EL = early luteal, ML = mid luteal, LL = late luteal, M = menstrual

^b Significance of Kruskal-Wallis test of means. ^c measurements are in 0.0046 mm² ^d measurements are in 0.121mm²

Table 7. 2 : The within group standard deviation and correlations between individual histological parameters examined for the whole series of specimens.

Histological parameters (a)	Mean	SD ^b														
		%GA	%VA	GD	VD	S	CG	IG	%C	VS	%V	%S	T	%Sm		
% glandular area	19.7	6.6	1.0													
% vascular space area	1.2	0.8	0.0	1.0												
Average glandular diameter	82.0	17.5	0.6	0.1	1.0											
Average vascular space diameter	18.1	6.6	0.0	0.7	0.1	1.0										
Stromal cell count ^c	64.2	19.6	-0.1	-0.1	-0.3	-0.3	1.0									
No. of complete glands ^d	2.1	0.8	0.1	-0.1	-0.4	-0.2	0.3	1.0								
No. of 'half' glands ^d	1.3	0.5	0.4	-0.1	-0.1	-0.1	0.1	0.2	1.0							
% gland with columnar lining	68.9	27	0.2	-0.1	0.2	0.0	-0.1	-0.2	0.0	1.0						
No. of vascular spaces ^d	3.2	1.9	-0.2	0.6	0.0	0.6	-0.2	-0.2	0.0	0.0	1.0					
% gland with vacuoles	35.1	35.2	0.2	0.2	0.2	0.1	-0.1	-0.1	-0.1	0.4	0.1	1.0				
% gland with secretions	77.4	28.2	0.2	0.0	0.2	0.0	0.0	-0.1	-0.1	0.1	0.0	0.3	1.0			
% gland with telescoping	0.09	0.2	0.3	0.2	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.0	1.0		
% gland with Smooth surface	20.1	21.7	-0.4	0.0	-0.4	-0.2	0.2	0.3	0.0	-0.2	-0.1	-0.2	-0.4	-0.1	1.0	

a %GA = total glandular area, % VA = total vascular space area, GD = average glandular diameter, VD = average vascular space diameter, S = stromal cell count, CG = No. of complete glands, IG = No. of incomplete glands, %C = % glands with columnar epithelial lining, VS = No. of vascular spaces, %V = % glands with vacuoles, %S = % glands with secretion, T = % glands with telescoping, %Sm = % glands with smooth epithelial lining. ^b SD = standard deviation, ^c measurements are in 0.0046 mm² ^d measurements are in 0.121mm²

Table 7.3: Standardised weights assigned to individual histological parameters along the first 4 linear discriminant functions.

Histological parameters	Linear Discriminant Functions			
	1	2	3	4
% glandular area	1.19	0.37	-0.75	0.25
% vascular space area	-0.34	-1.02	0.34	-0.19
Average glandular diameter	-0.51	-0.12	-0.10	-0.62
Average vascular space diameter	0.38	0.13	-0.11	-0.30
Stromal cell count	0.14	-0.01	-0.25	-0.09
No. of complete glands	-0.29	-0.27	0.34	-0.14
No. of 'half' glands	0.15	0.14	0.59	0.13
% gland with columnar lining	-0.06	-0.46	0.78	0.13
No. of vascular spaces	0.21	0.20	-0.11	0.49
% gland with vacuoles	0.37	0.68	0.01	-0.53
% gland with secretions	-0.02	-0.33	-0.27	0.78
% gland with telescoping	-0.00	0.51	0.11	0.08
% gland with Smooth surface	0.33	-0.14	-0.30	-0.12

Table 7.4 :Mean Scores on the first four linear discriminant functions, in the 4 dose groups of trimegestone compared to the natural cycle.

	Trimegestone dose group (mg)				P	Natural Cycle ^a			
	0.05	0.1	0.25	0.5		EL	ML	LL	M
	n=23	21	29	26	8	8	5	8	8
LDF 1 ^b	-0.71	-0.01	-0.44	-0.35	-0.82	2.82	1.15	1.63	0.11
LDF 2	-0.09	0.25	0.64	0.55	-1.77	0.67	-1.40	-0.42	-1.95
LDF 3	-0.19	-0.14	-0.20	0.22	1.58	0.92	-1.97	-0.17	-0.25
LDF 4	0.23	0.12	0.33	-0.70	0.61	-0.24	-0.01	0.89	-1.06

^a P = proliferative, EL = early luteal, ML = mid luteal, LL = late luteal, M = menstrual.

^b LDF = linear discriminant function

Table 7.5 : Standardised weights for the rotated scales shown in figure 7. 4c

Histological parameters	Rotated Linear Discriminant Functions	
	1	2
% glandular area	0.92	0.84
% vascular space area	0.71	-0.81
Average glandular diameter	-0.14	-0.50
Average vascular space diameter	0.07	0.40
Stromal cell count	0.08	0.12
No. of complete glands	0.09	-0.39
No. of 'half' glands	-0.04	0.20
% gland with columnar lining	0.37	-0.28
No. of vascular spaces	-0.07	0.28
% gland with vacuoles	-0.40	0.67
% gland with secretions	0.28	-0.18
% gland with telescoping	-0.44	0.25
% gland with Smooth surface	0.29	0.21

Chapter Eight

Endometrial Leukocytes

Introduction

The major cell population of leukocyte in the endometrium, as defined by leukocyte common antigen (LCA), CD45⁺ cells are the macrophages, T-lymphocytes (T-cells) and endometrial granular lymphocytes (eGL)^{147 194}. CD45⁺ cells are scattered in the stroma, around the glands and around the blood vessels¹⁵⁰. In the proliferative phase, CD2⁺ CD3⁺ T-cells are the predominant cells, while in the luteal phase, CD56⁺ CD2⁺ CD38⁺ CD16⁻ CD3⁻ lymphocytes predominate^{195 196}.

The macrophages are bone marrow derived cells and comprise about 50% of CD45⁺ cells^{197 198 199}. They are responsible for phagocytosis, antigen processing and release of cytotoxic mediators such as interleukin-1 (IL-1), TNF α and TGF- β ¹⁵¹. The macrophage population in the stroma increases from day LH+4 to LH+13, where it is primarily responsible for the 62% increase in total leukocytes, but mainly between days LH+10 - LH+13 by about 28%¹⁵⁰. In another report, macrophages comprised 6-15% of the total cells in the functionalis on days 27-28 of the natural cycle²⁰⁰.

T-cells account for about 34% of CD45⁺ cells. The ratio of CD8⁺/CD4⁺ is 2.8 during the luteal phase, which is the reversal of the ratio in the blood¹⁵⁰. The number of T-cells increases post-ovulation from day LH+4 to LH+13, however, it is higher in the early luteal phase compared to late luteal phase¹⁵⁰. About 70% of CD2⁺ CD3⁺ T-cells are CD8⁺, while about 25% are CD4⁺ and the ratio of CD8⁺ : CD4⁺ increases with the

progress of the endometrium from proliferative to menstrual phases^{197 198 199}. While the peripheral blood CD3⁺ cells are active during the whole natural cycle, endometrial T-cells are cytolytically active only during the proliferative phase compared to the luteal phase, probably due to the effect of progesterone²⁰¹. A subset of T-cells express interferon γ (INF- γ)⁺²⁰², and B lymphocytes are also found in the endometrium and they produce IgA¹⁵¹.

Endometrial granular lymphocytes (eGL), account for about 50% of endometrial leukocytes, while in the peripheral blood, CD56⁺ CD16⁻ lymphocytes account for less than 2% of the total lymphocytes¹⁹⁵. The function of eGL is not clear, but may be related to implantation, in addition, CD56⁺ CD16⁻ eGL have NK-cell like cytotoxic activity comparable to CD56⁺ CD16⁺ NK-cells from peripheral blood²⁰³.

A differential increase of CD56⁺ CD16⁺ cell was observed in the basalis zone compared to the functionalis zone of the endometrium, but the difference was not statistically significant²⁰⁴. CD56⁺ cell count showed no difference in level between day LH+4 to LH+7, but there is an increase in their proliferative activity between day LH+7 to LH+13¹⁵⁰. These cells comprised 15% to 25% of the total number of cells in the stroma during the luteal phase^{205 195}.

Mast cells are present in the endometrium throughout the natural cycle, and these cells exist in 2 phenotypes; mucosal mast cells which are immunopositive to the proteinase-tryptase, and the connective tissue mast cells that contain tryptase and chymase enzymes. Mast cells

may have a role in tissue shedding at menstruation since tryptase release can establish a cascade of MMPs activation ¹⁴⁶.

The increase of 20-25% of leukocyte cell population in late luteal phase is predominantly due to the increase in the number of macrophages, while the massive increase in the menstrual phase is mainly due to neutrophils ¹⁴⁷. Some neutrophils are immunopositive to IFN- γ , which has a role in macrophage activation ^{202 206}.

Eosinophils are also present in the endometrium but mainly premenstrually, where there is an increase in their number ¹⁴⁶. Many endometrial leukocytes express MMP-9, such as neutrophils, T-cells and macrophages, hence may be involved in endometrial shedding ^{207 208}.

The increase in the number of stromal leukocytes which appears from early luteal phase onwards may be modulated by progesterone ^{148 149}.

However, the effect of progesterone and probably oestrogen, on the endometrial leukocytes is most probably indirect, as progesterone and oestrogen receptors are not detected on the leukocytes ^{209 210}.

Endometrial chemokines, such as MCP-1 and RANTES (RANTES = regulated upon activation normal T-cell expressed and secreted) have been implicated as possible leukocytes attractants ^{211 212}.

The origin of these leukocytes has been disputed. Whether they migrate from the circulation under the effect of chemotactic factors

produced by stromal cells, or whether they proliferate locally within the endometrium at the beginning of the luteal phase is not known. Ki-67 is a non-histone proliferation associated antigen, which is used to assess proliferation as it is not degraded during apoptosis. Moreover, endometrial expression of ki-67 antigen appears to be modulated during the natural cycle, probably by the direct influence of sex steroid⁹².

Aim

I examined the leukocytic population and the proportion of proliferating leukocytes in the endometrium of postmenopausal women treated with the 4 doses of trimegestone-based HRT compared to the natural cycle, in an attempt to identify factors that characterise the effect of trimegestone on the endometrium. The possibility of correlating the changes in these leukocyte markers with different bleeding patterns is also examined.

Women and method

The study population of 176 women are described in page 51 –52. Endometrial biopsies were obtained on day 24 of the last treatment cycle. The endometrial samples were fixed immediately in 10% formal-saline embedded in paraffin and 4 µm sections were stained with H&E for histological assessment (standard protocol at histopathology department, Leicester Royal Infirmary).

Treatment

Women were randomised to receive one of 4 doses of trimegestone

Page 90.

The control

The characteristics of the control samples (n=28) are described in page 53. The histological diagnoses, were proliferative (n= 6), early luteal (n= 7), late luteal (n= 7) and menstrual (n= 8).

Immunohistochemistry

The techniques for single labelling of CD45, CD56, CD3, ki-67, and for the double labelling of ki-67/CD45 are described in page 55-60.

Assessment of the staining

I have assessed the pattern of the distribution of the positively stained cells for all the antigens used in the endometrial slides, and then I have captured the images of ten randomly selected fields ¹⁶⁸ per slide (x200) to evaluate the endometrial cells positive for CD45, CD56, CD3 and ki-67 antigens 63. In the doubly labelled sections ki-67/CD45 and the control endometrial specimens, 15 randomly selected fields per slide were captured. Images were captured using Axioplan microscope (Carl Zeiss, Herts UK), and a colour video camera (Sony CCD/RGB); each image (x200) covers an area of 0.121 mm² using an eye piece graticule. The images were displayed on RGB monitor and the positive cells were counted using the KS300 image analysis programme (Kontron Imaging

Systems, Thame, UK). Cell counts were restricted to the functionalis layer.

Statistics

Cellular count in all randomised fields, for each marker used, were analysed using a linear discriminant analysis models based on 8 groups, viz.: the 4 doses of the trimegestone-treated specimens, and the 4 phases of the endometrium of the natural cycle, proliferative, early luteal, late luteal and menstrual (GENSTAT 5.3 package) ^{179 193}.

Unpaired t-test was used to compare the cell count of specimens obtained from women who bled by the day of the biopsy to those who had not bled by then. Based on assumed standard deviation of 40 for the CD45 cell count, and 20 for the CD3 and CD56 cell counts, these tests have 80% power to detect mean difference at $\alpha = 0.05$ of 26 and 13, respectively.

Results

In the MRU, Leicester, 176 women were randomised to one of the 4 dose groups and 131 completed the study, their demographic data are as described in page 126. Two women declined to have a biopsy at the end of the study, and therefore the total number of endometrial samples obtained at the end of the study was 129.

The total number of endometrial specimens from trimegestone-treated women subjected for immunohistochemical analysis in this study was less than the total number of biopsies collected at the end of the 6 months

treatment. This is due to the fact that **a.** 12 specimens from women who bled before the day of the biopsy were excluded (page 142). **b.** some samples were scanty and **c.** other samples could not sustain the process of antigen retrieval and disintegrated. The number of evaluable sections therefore, were 102, 109, 110, 110, 113 for CD45, CD56, CD3, ki-67 and ki-67 /CD45 antigens, respectively, Figure 8.1.

a. Endometrium of the natural cycle

CD45⁺, CD56⁺ and CD3⁺ stromal cells were scattered in the endometrium with aggregations around the glands (Figure 8.2a, 8.2b, 8.2c and 8. 2d). The number of these leukocytes was higher in the functionalis compared to the basalis. The leukocyte counts (CD45⁺, CD56⁺ and CD3⁺ cells) were low in the proliferative and early luteal phases, but were markedly increased in the late luteal and menstrual phases (Table 8.1a, 8.1b & 8.1c, Figure 8.3a). ki-67⁺ cells were present in the endometrial glands but more frequently expressed in the stroma (Figure 8.2e), and were generally more prevalent in the functionalis layer than the basalis. The ki-67 antigen expression in the glands was raised in the proliferative and early luteal phases, but declined in the late luteal and menstrual phases (Table 8.1d, Figure 8.3b). In the stroma, Ki-67 expression remained relatively constant during the different phases of the natural cycle.

The total number of doubly labelled ki-67⁺/CD45⁺ cells showed a similar pattern of expression to their counterpart sections which were singly labelled for either CD45 or ki-67 antigen, in terms of stromal

scatter and around glands aggregations (Figure 8.2f, 8.2g and Figure 8.3c). Glandular expression of ki-67⁺ in doubly labelled sections was similarly distributed compared to sections singly labelled for this antigen, however, the actual number of ki-67⁺ and CD45⁺ cells was generally lower in the doubly labelled sections. Of the total CD45⁺ cell count, only 13% were also positive for ki-67 antigen (Table 8.1e).

b. Trimegestone treated endometrium

The mean count for CD45⁺, CD56⁺ and CD3⁺ cells in the endometrial biopsies obtained from trimegestone-treated women was not statistically significantly different between the 4 dose groups. However, endometrial sections from women who bled by the day of the biopsy contained higher number of CD45⁺ (P=0.043) and CD56⁺ cells (unpaired t-test; P=0.018), compared to sections from women who have not bled by then. There was no statistically significant difference for CD3⁺ cell count between these 2 groups.

To visualise the relationship between the findings for the treated groups, the 4 doses of trimegestone, and the four phases of the endometrium of the natural cycle, I have adopted the method of linear discriminant analysis (page 145). This represents multi-dimensional count data in two dimensions chosen to best distinguish between the groups (Tables, 8.2a-e).

Figure 8. 4a shows the results for the CD45⁺ cells. Three counts were made from the fields; cells around the glands, cells in the stroma and intra-epithelial lymphocyte (IEL) cells. The two dimensional plot captures 96% of the discriminant information. The profiles of counts in the treated groups were similar to those for the proliferative and early luteal phases of the natural cycle and were distinguished from the endometrium of the late luteal and menstrual phases of the natural cycle mainly due to CD45⁺ cell count around the glands.

The results for CD56⁺ cells (Figure 8. 4b) were similar to those for CD45⁺ cells with the treated groups resembled the proliferative and early luteal phases of the natural cycle. The discrimination occurs mainly along the first function for which a high value is associated with high stromal and IEL counts. The CD56⁺ cell count around the glands does not help distinguish the groups. CD3⁺ cell count (Figure 8. 4c) was similar but was less clear with only the menstrual phase well distinguished from the treated groups.

The ki-67⁺ cells were counted in the glands and the stroma (Figure 8. 4d). In this case the proliferative and early luteal phases were distinguished from the treated groups because of the ki-67⁺ cells in the glands.

Finally, the number of Ki-67⁺ /CD45⁺ doubly labelled cells (Figure 8. 4e) has produced six counts: CD45⁺ cells: around the glands, scattered in the stroma, and the IEL cells, ki-67⁺ cells: in the glands and stroma, and the number of ki-67⁺ /CD45⁺ cells in the stroma. The two dimensional plot reproduces 85% of the discrimination and shows the treated groups to be similar to the menstrual phase of the natural cycle, with the late luteal phase of the natural cycle distinguished because of a combination of higher Ki-67⁺ cell counts in the glands and stroma, higher Ki-67⁺ /CD45⁺ cell count and lower CD45⁺ cell counts around the glands. The proliferative and early luteal phases were distinguished by the gland counts in ki-67⁺ and to a lesser extent in CD45⁺ cells.

Endometrial sections from women who bled by the day of the biopsy contained higher number of CD45⁺ and CD56⁺ cells compared to sections from women who have not bled by then.

Summary of the study

In this study I used immunohistochemical techniques to assess the distribution of ki-67 and 3 markers of leukocyte in the endometrium of the trimegestone-based HRT compared to that of the natural cycle.

There was an increase in the endometrial leukocytes in late luteal and menstrual phases.

Glandular expression of ki-67 increased in the proliferative and early luteal phases, while in the stroma, there was no change between the different phases of the natural cycle.

There was no dose-response effect of trimegestone on the distribution of endometrial leukocytes. However, given the inter-subjective variability, the number of endometrial specimens in each dose group may not be adequate to show subtle differences. Women who bled on the day of the biopsy had a higher count of CD45 and CD56 compared to women who had not bled by then.

This study showed that the increase in the endometrial leukocytes in the late luteal phase is due to cellular migration rather than proliferation, since 6:7 of the cells were negative to ki-67.

In order to explore differences between the eight sets of endometrial biopsies, I adopted the linear discriminant analysis. With this technique I found that the expression of CD45⁺, CD56⁺ and CD3⁺ cells in the trimegestone-treated endometrium was similar to the proliferative and early luteal phases of the natural cycle. The expression of the proliferating marker Ki-67 and the proliferating doubly labelled leukocytes (Ki-67⁺ / CD45⁺) cells were similar to the menstrual phase endometrium of the natural cycle.

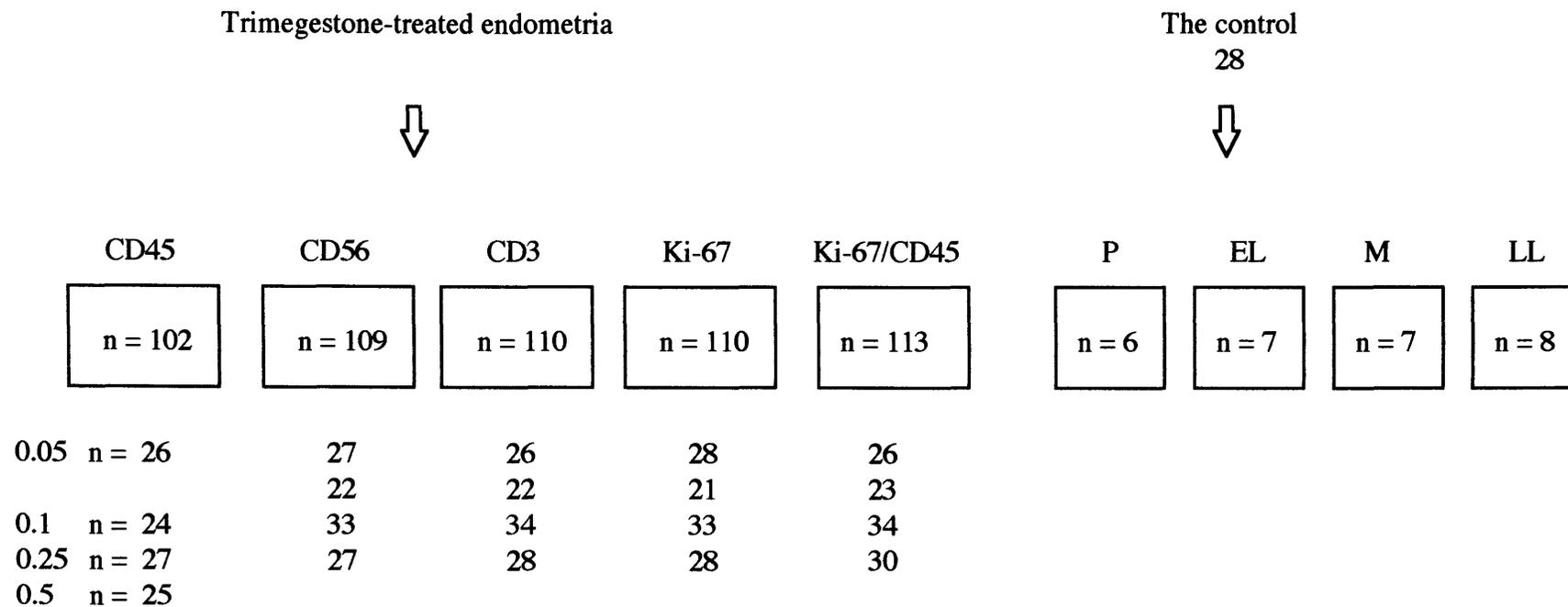
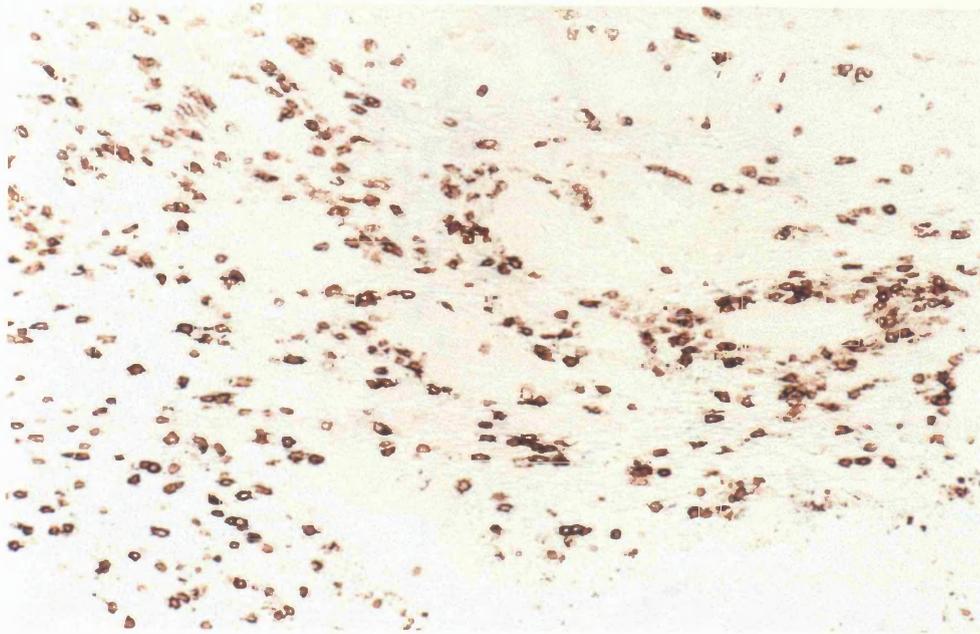
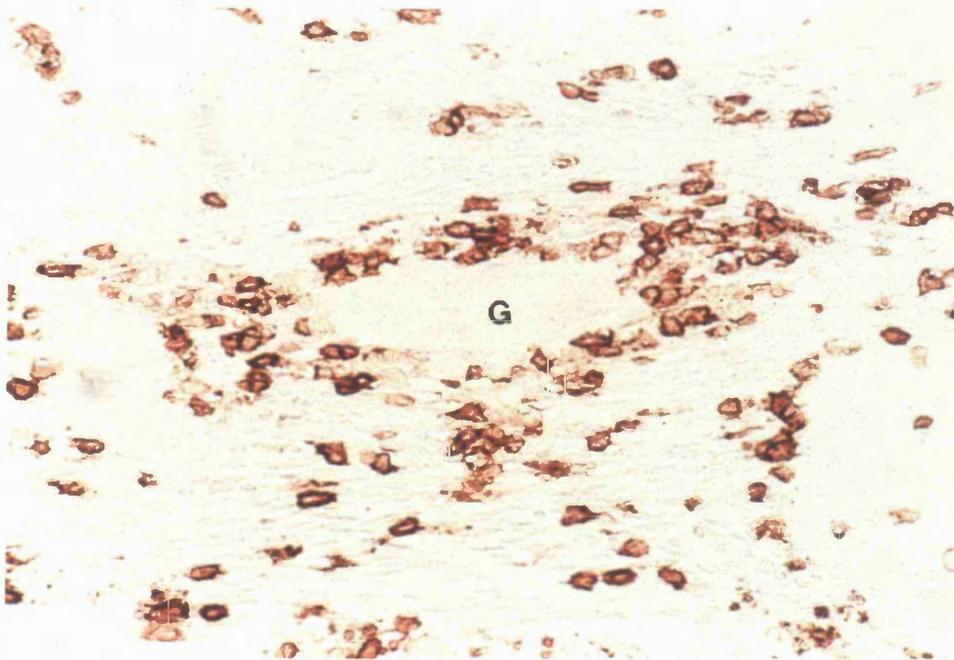


Figure 8.1. Number of specimens in the Endometrial Leukocyte study. Women who bled before the day of the biopsy were excluded (n = 12), P = proliferative, EL = early luteal, LL = late luteal & M = menstrual.

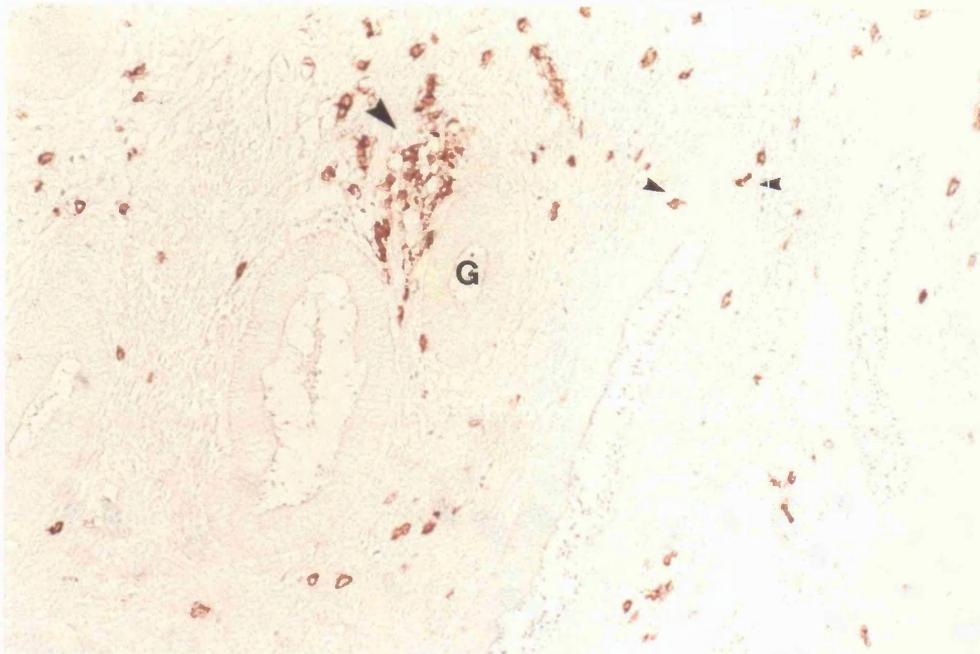


8.2a

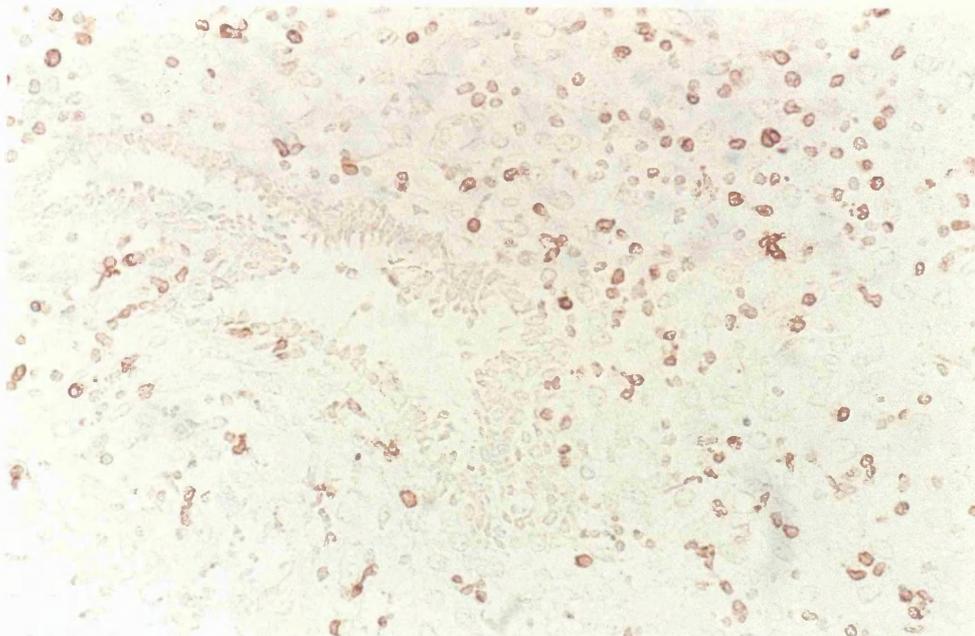


8.2b

Figure 8.2a & 2b: Immunohistochemical staining of endometrial sections in the menstrual phase, showing the distribution of CD45⁺ cells in the stroma, original magnification x 200, 1cm = 100 μ m (8.2a). The distribution of CD45⁺ cells in the stroma with an aggregation around the gland, original magnification x 400, 1cm = 50 μ m, G= gland (8.2b).

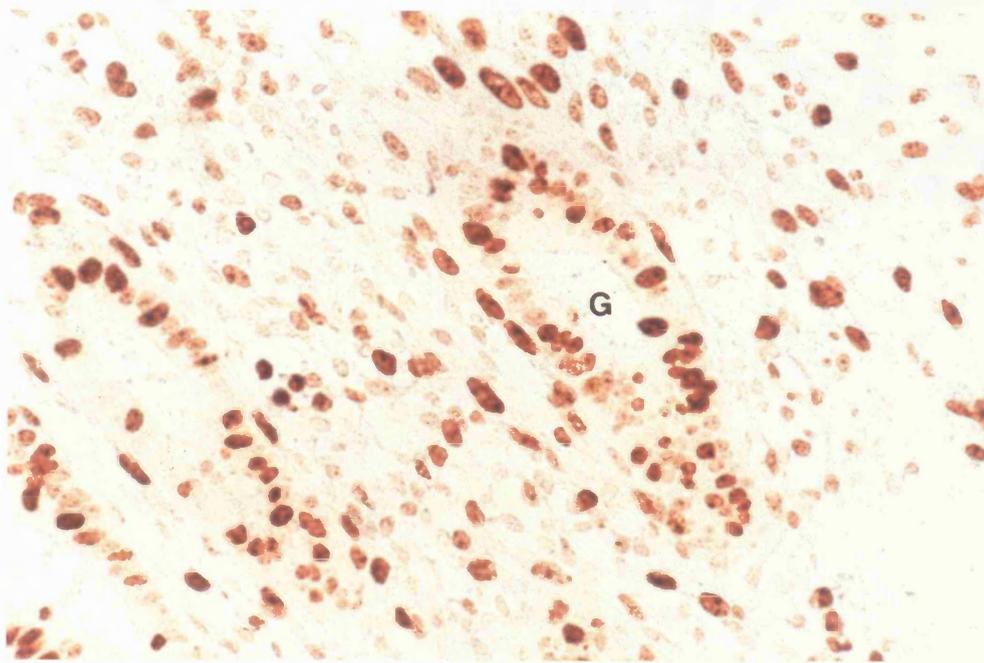


8.2c



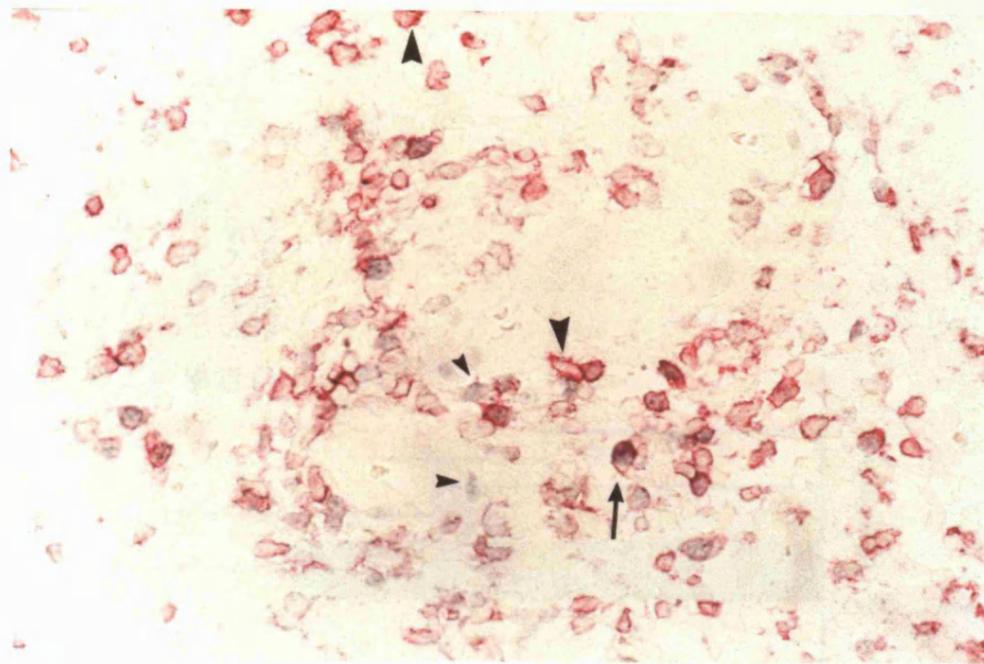
8.2d

Figure 8.2c & 2d: Immunohistochemical staining showing CD56⁺ cells in the stroma in the proliferative phase around the gland (large arrow head) and IEL cells (small arrow head), original magnification x 200, 1cm = 100 μ m (2c). Immunohistochemical staining of endometrial sections in the menstrual phase showing the distribution of CD3⁺ cells in the stroma, original magnification x 400, 1cm = 50 μ m (2d).

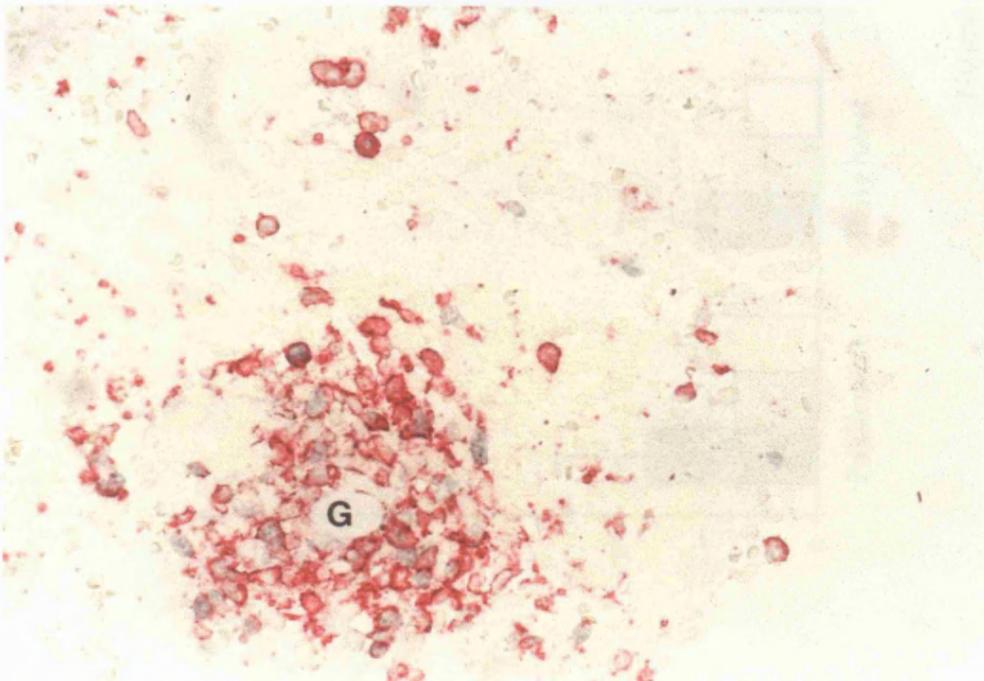


8.2e

Figure 8.2e: Immunohistochemical staining of endometrial section in the proliferative phase showing the distribution of ki-67 nuclear staining of the glands and stroma., original magnification x 200, 1cm = 100 μ m. G = Gland.



8.2f



8.2g

Figure 8.2f & 2g: immunohistochemical staining of endometrial sections in the HRT cycle showing the distribution of Double labelling, CD45⁺ cells (large arrow head), Ki-67⁺ cells (small arrow head) and CD45⁺/Ki-67⁺ cells (arrow) in the stroma , original magnification x 200, 1cm = 100 μ m (1f). CD45⁺/Ki-67⁺ cells with an aggregation around the gland, original magnification x 200, 1cm = 100 μ m (1g). G = Gland.

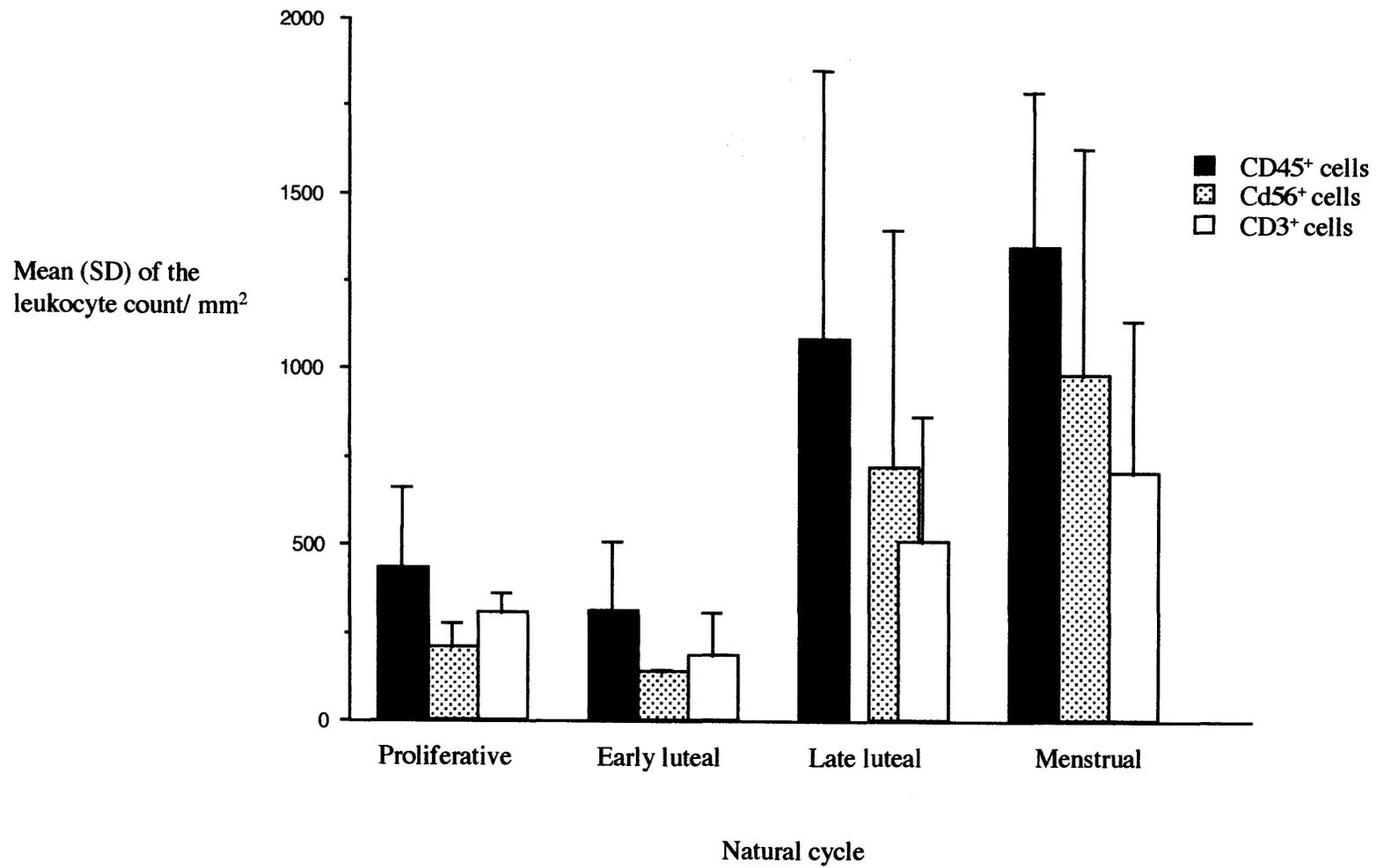


Figure 8.3a: The distribution of the stromal endometrial leukocytes in the natural cycle.

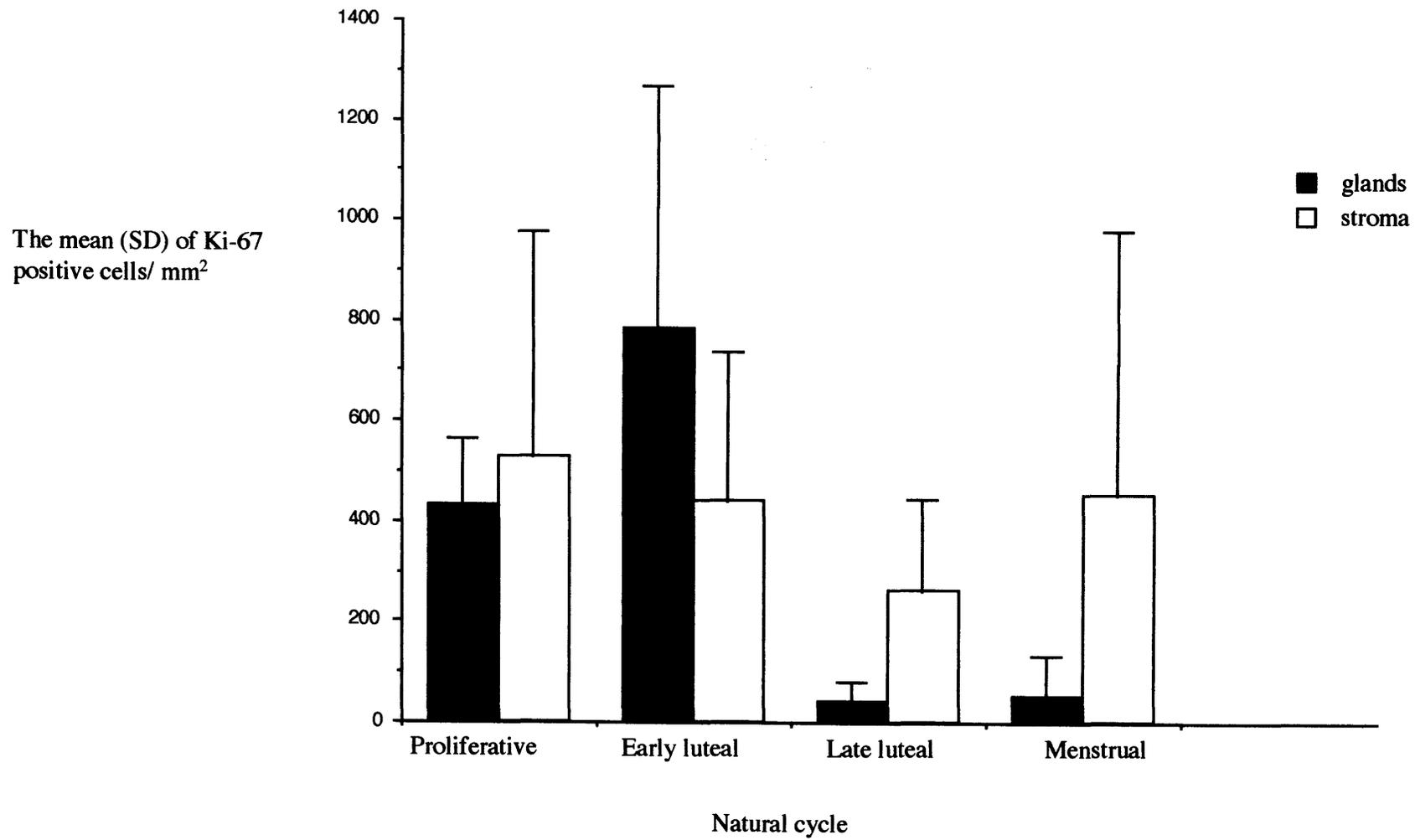


Figure 8.3b : The distribution of Ki-67⁺ cells in the endometrial glands and stroma in the natural cycle.

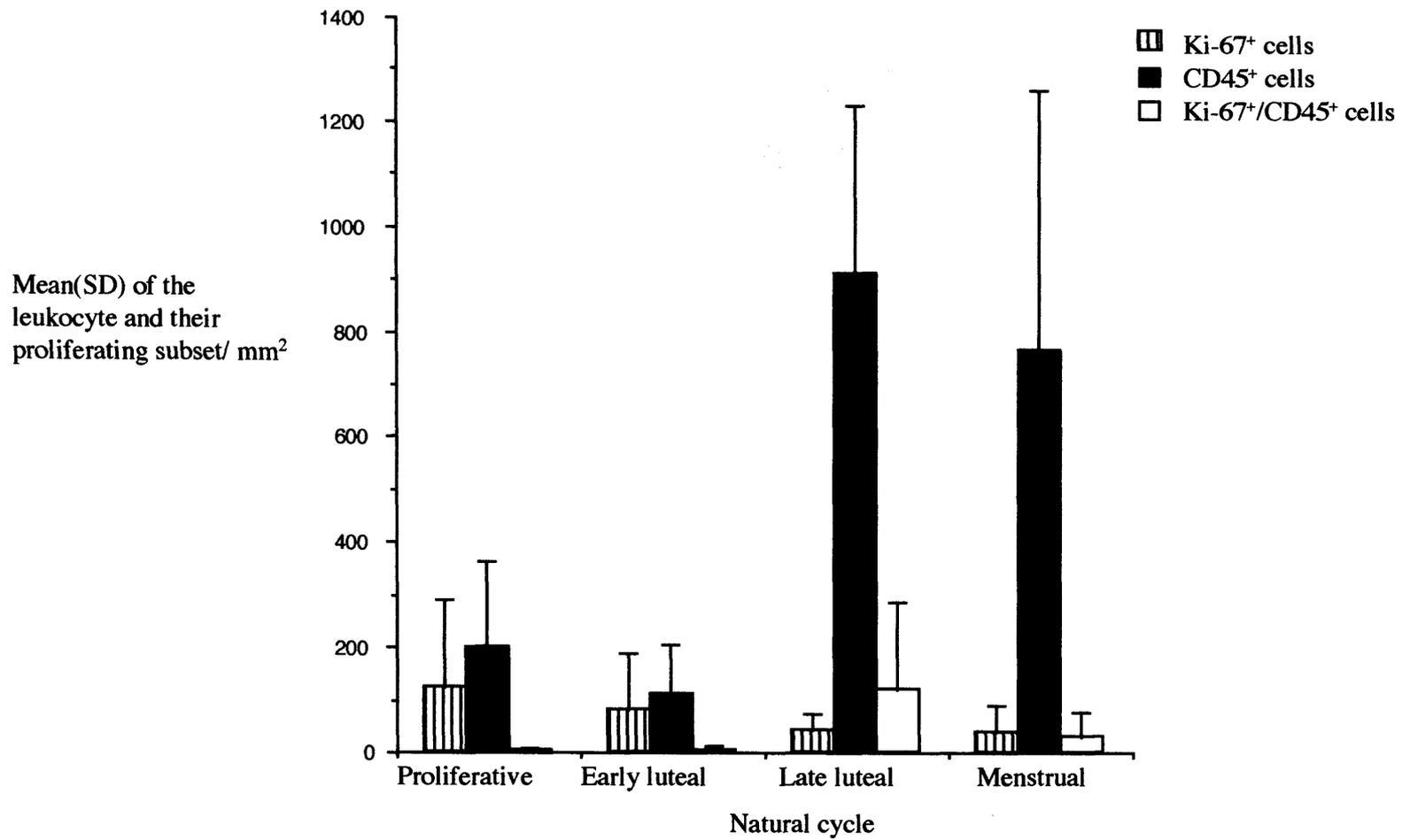


Figure 8.3c: The distribution of CD45⁺ cells and its proliferating subset in the endometrial stroma in the natural cycle.

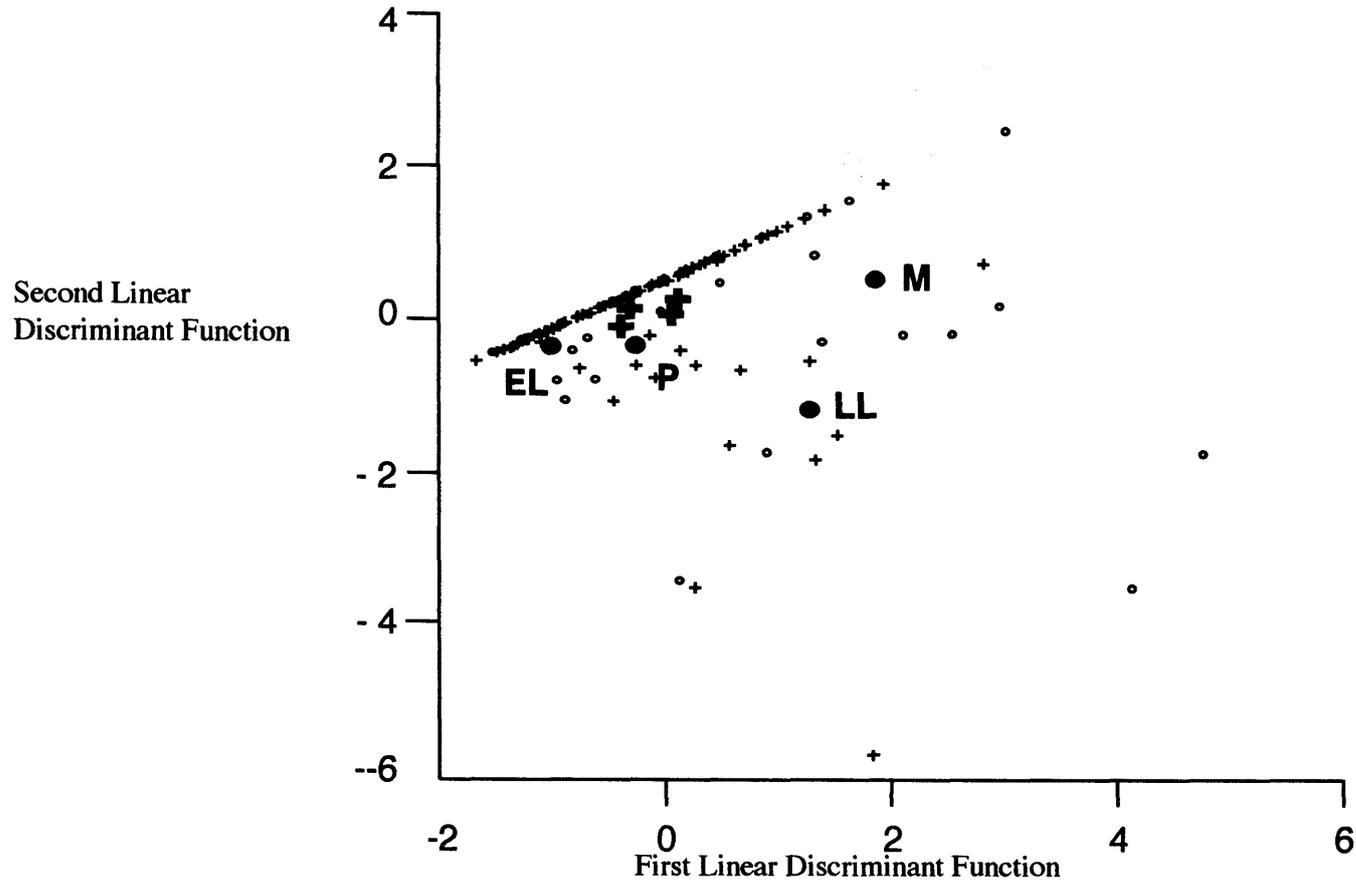


Figure 8.4a : Linear discriminant analysis showing the distribution of CD45⁺ cells in the trimegestone-treated endometrium and their relation to the phases of the natural cycle. Light crosses represents the mean cell count in the treated individual woman and the heavy crosses represent the group means. Light circles represent the mean cell count in the individual women in the control group and the heavy circles represent the group means. P= proliferative, EL= early luteal, LL= late luteal, M= menstrual.

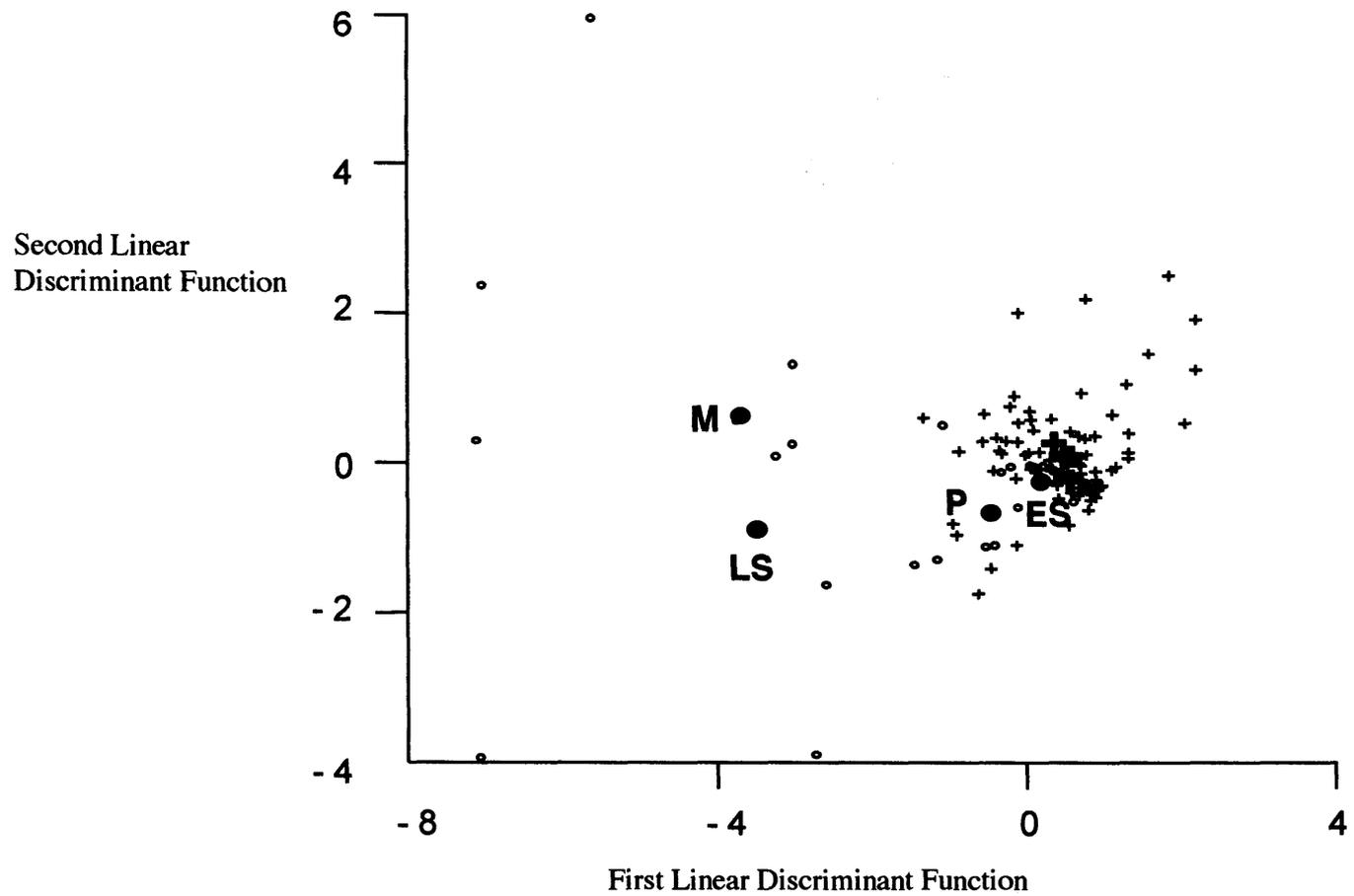


Figure 8.4b : Linear discriminant analysis showing the distribution of CD56⁺ cells in the trimegestone-treated endometrium and their relation to the phases of the natural cycle. Light crosses represents the mean cell count in the treated individual woman and the heavy crosses represent the group means. Light circles represent the mean cell count in the individual women in the control group and the heavy circles represent the group means. P= proliferative, EL= early luteal, LL= late luteal, M= menstrual.

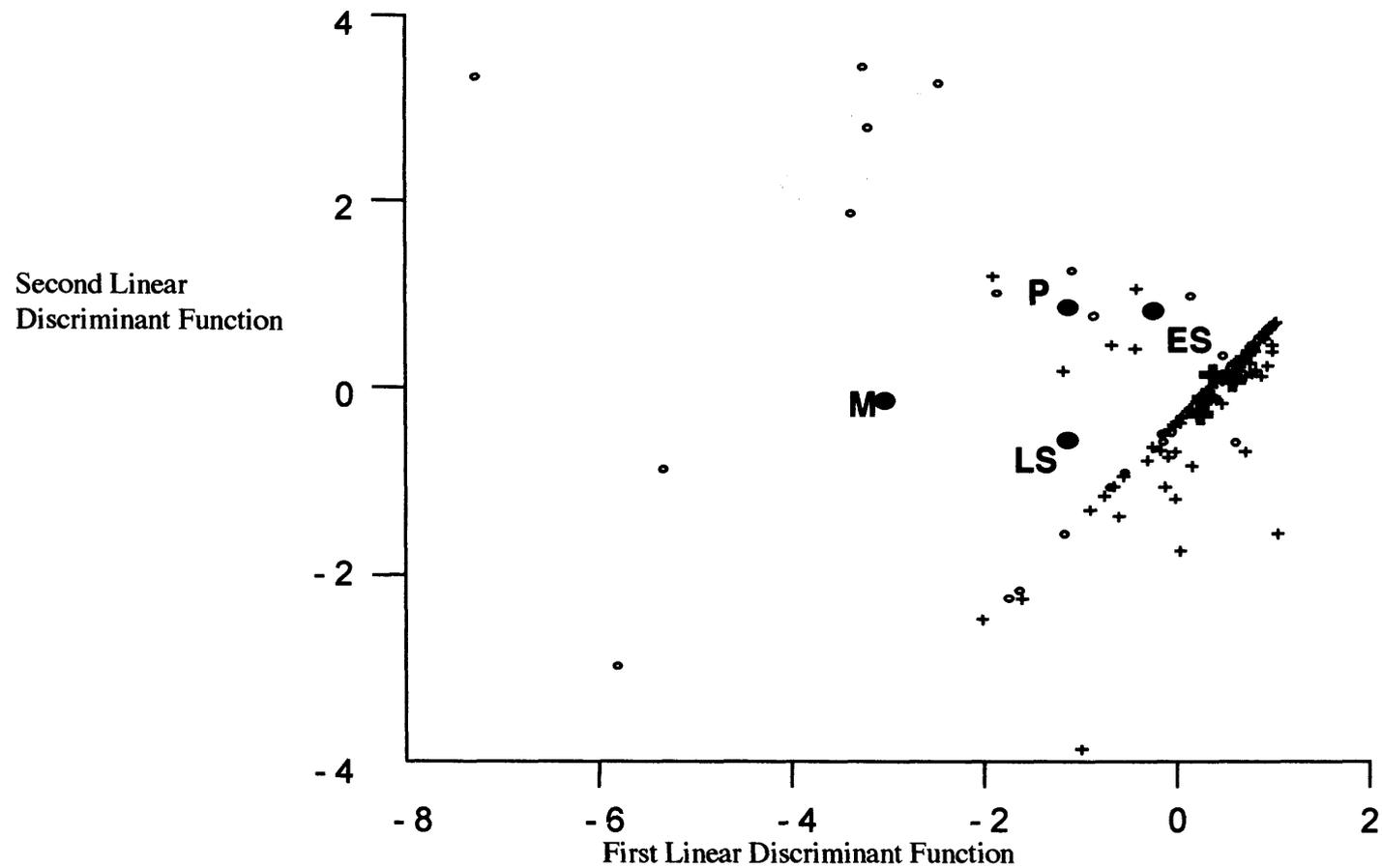


Figure 8.4c : Linear discriminant analysis showing the distribution of CD3⁺ cells in the trimegestone-treated endometrium and their relation to the phases of the natural cycle. Light crosses represents the mean cell count in the treated individual woman and the heavy crosses represent the group means. Light circles represent the mean cell count in the individual women in the control group and the heavy circles represent the group means. P= proliferative, EL= early luteal, LL= late luteal, M= menstrual.

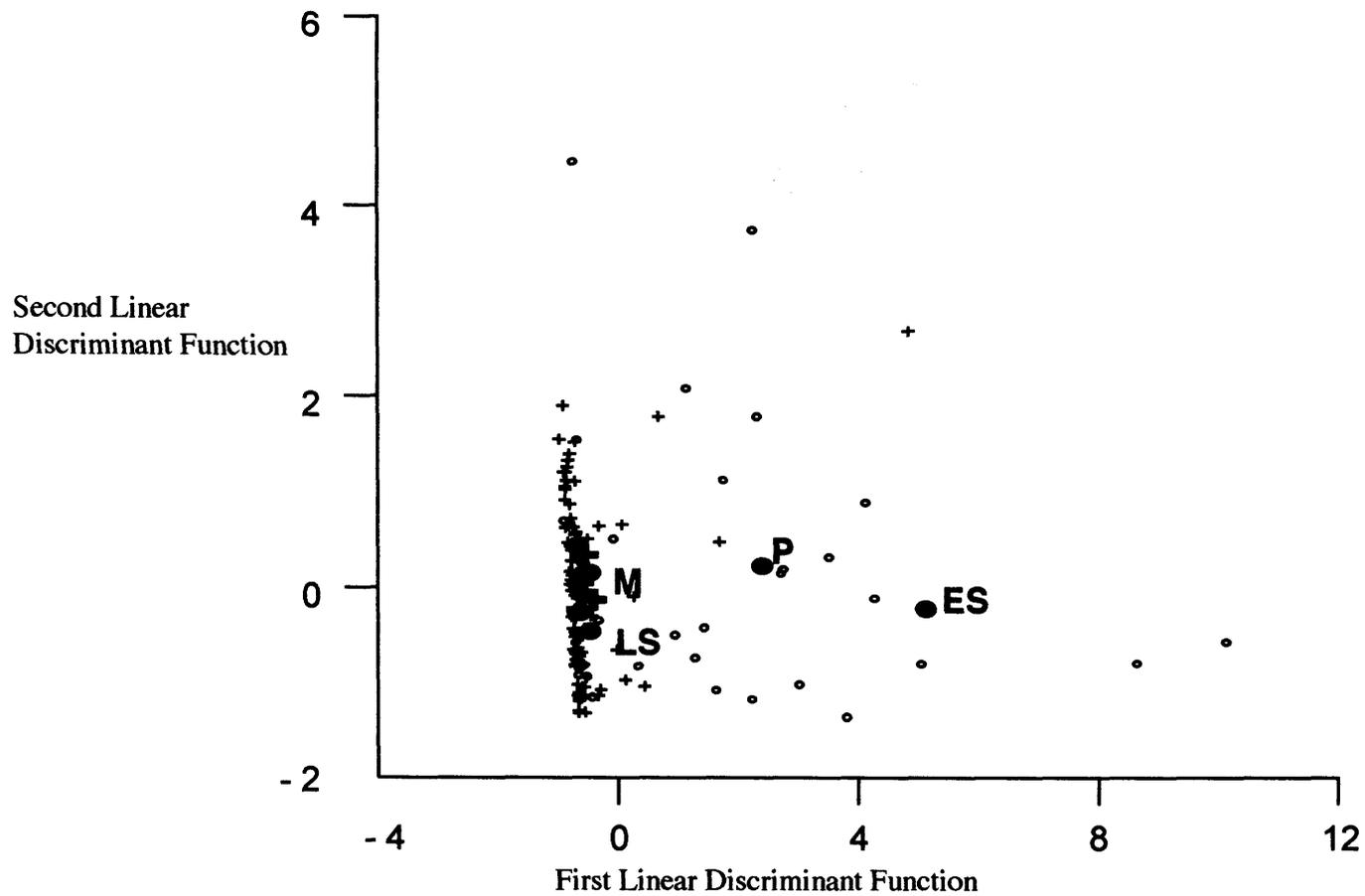


Figure 8.4d : Linear discriminant analysis showing the distribution of ki-67⁺ cells in the trimegestone-treated endometrium and their relation to the phases of the natural cycle. Light crosses represents the mean cell count in the treated individual woman and the heavy crosses represent the group means. Light circles represent the mean cell count in the individual women in the control group and the heavy circles represent the group means. P= proliferative, EL= early luteal, LL= late luteal, M= menstrual.

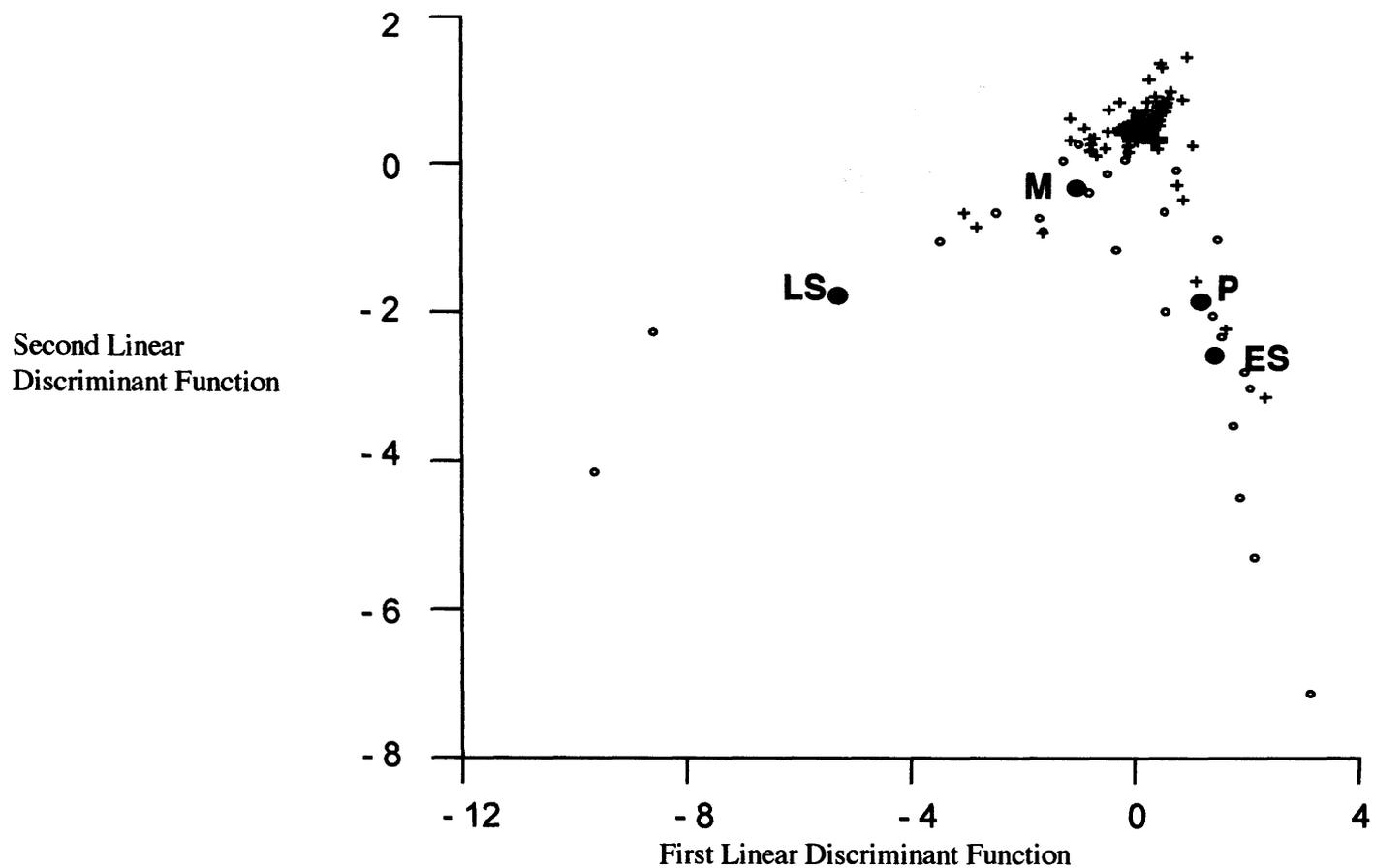


Figure 8.4e : Linear discriminant analysis showing the distribution of ki-67⁺ / CD45⁺ cells in the trimegestone-treated endometrium and their relation to the phases of the natural cycle. Light crosses represents the mean cell count in the treated individual woman and the heavy crosses represent the group means. Light circles represent the mean cell count in the individual women in the control group and the heavy circles represent the group means. P= proliferative, EL= early luteal, LL= late luteal, M= menstrual.

Table 8.1a : Mean and standard deviation (SD) of CD45⁺ cells scattered in the stroma, around the glands and intra epithelial leukocytes (IEL), in the endometrium treated with sequential trimegestone and in the natural cycle. Data are per mm² endometrial tissue.

	Trimegestone dose groups (mg)				P	Endometrium of the natural cycle ^a		
	0.05	0.1	0.25	0.5		EL	LL	M
Mean (SD)								
CD45	n = 26	24	27	25	6	6	7	7
Stroma	583.7 (349.7)	805.3 (495.8)	692.9 (332.7)	515.8 (295.2)	410.3 (201.9)	308.7 (188.5)	1011.7 (707.3)	1302.4 (440.2)
Around the glands	5.9 (17.1)	12.3 (26.1)	8.4 (19.0)	9.2 (37.2)	21.3 (27.5)	6.3 (11.0)	64.5 (73.1)	31.1 (29.6)
IEL	9.3 (10.1)	13.7 (15.7)	14.7 (16.2)	13.6 (14.2)	14.8 (19.4)	4.5 (5.6)	22.1 (28.3)	26.3 (36.7)

^aP= Proliferative, EL= Early luteal, LL= Late luteal, M= Menstrual.

Table8.1b: Mean and standard deviation (SD) of CD56⁺ cells scattered in the stroma, around the glands and intra epithelial leukocytes (IEL), in the endometrium treated with sequential trimegestone and in the natural cycle. Data are per mm² endometrial tissue.

	Trimegestone dose groups (mg)				P	Endometrium of the natural cycle ^a		
	0.05	0.1	0.25	0.5		EL	LL	M
Mean (SD)								
CD56	n = 27	22	33	27	6	5	5	8
Stroma	127.0 (149.1)	236.5 (196.8)	148.1 (131.8)	147.5 (130.9)	202.2 (70.0)	136.7 (59.3)	707.6 (650.9)	955.7 (604.5)
Around the glands	17.9 (31.5)	70.9 (218.7)	24.6 (39.0)	17.5 (29.8)	0.8 (2.02)	0 (0.0)	3.8 (5.4)	21.0 (46.3)
IEL	3.6 (9.6)	2.4 (5.2)	1.9 (2.7)	1.4 (2.7)	8.1 (8.4)	1.8 (2.6)	21.9 (24.1)	13.3 (15.6)

^a P= Proliferative, EL= Early luteal, LL= Late luteal, M= Menstrual.

Table 8.1c : Mean and standard deviation (SD) of CD3⁺ cells scattered in the stroma, around the glands and intra epithelial leukocytes (IEL), in the endometrium treated with sequential trimegestone and in the natural cycle. Data are per mm² endometrial tissue.

	Trimegestone dose groups (mg)				P	Endometrium of the natural cycle ^a		
	0.05	0.1	0.25	0.5		EL	LL	M
Mean (SD)								
CD3	n = 26	22	34	28	6	5	5	8
Stroma	292.9 (266.9)	164.3 (120.4)	256.3 (176.1)	183.0 (142.6)	283.1 (63.9)	170.7 (126.3)	496.4 (356.3)	670.8 (421.6)
Around the glands	0.06 (0.3)	0.0 (0.0)	0.9 (3.2)	0.8 (3.3)	18.0 (17.6)	10.6 (19.3)	9.3 (19.4)	28.0 (34.0)
IEL	4.2 (7.7)	2.8 (9.5)	6.3 (17.9)	2.1 (3.2)	2.2 (0.9)	1.1 (0.7)	6.3 (6.3)	10.2 (25.9)

^a P= Proliferative, EL= Early luteal, LL= Late luteal, M= Menstrual.

Table 8.1d : Mean and standard deviation (SD) of ki-67⁺ cells in the endometrium treated with sequential trimegestone and in the natural cycle. Data are per mm² endometrial tissue.

	Trimegestone dose groups (mg)				P	Endometrium of the natural cycle ^a		
	0.05	0.1	0.25	0.5		EL	LL	M
Mean (SD)								
Ki-67	n = 28	21	33	28	13	5	4	10
Glands	22.9 (31.3)	37.3 (73.0)	25.3 (31.7)	48.5 (148.5)	426.7 (132.4)	662.0 (432.5)	45.5 (43.1)	50.9 (79.7)
Stroma	363.4 (290.8)	532.7 (230.9)	419.0 (261.8)	362.8 (293.5)	532.8 (443.4)	448.4 (325.4)	293.3 (186.7)	449.0 (520.6)

^aP= Proliferative, EL= Early luteal, LL= Late luteal, M= Menstrual.

Table 8.1e : Mean and standard deviation (SD) of ki-67⁺/CD45⁺ cells in the endometrial tissue treated with sequential trimegestone and in the natural cycle. Data are per mm² endometrial tissue.

	Trimegestone dose groups (mg)				P	Endometrium of the natural cycle ^a		
	0.05	0.1	0.25	0.5		EL	LL	M
Mean (SD)	n = 26	23	34	30	13	4	4	10
Ki-67 (glands)	7.0 (25.0)	3.6 (9.9)	3.5 (9.7)	17.5 (55.1)	146.8 (111.5)	190.2 (211.0)	14.6 (16.4)	9.1 (16.2)
Ki-67 (stroma)	50.8 (53.1)	97.7 (97.5)	89.5 (81.0)	70.4 (99.8)	120.8 (167.0)	80.3 (105.8)	40.2 (31.8)	36.9 (51.4)
CD45 (stroma)	197.5 (156.8)	269.6 (197.3)	195.6 (172.3)	224.4 (448.7)	200.6 (156.2)	111.7 (90.4)	787.3 (214.9)	742.7 (494.8)
CD45 (around glands)	0.0 (0.0)	4.7 (15.1)	3.2 (14.2)	0.7 (2.3)	0.5 (1.5)	0.0 (0.0)	115.0 (116.9)	13.4 (25.2)
CD45 (IEL)	5.0 (6.2)	3.8 (6.9)	5.5 (8.2)	6.8 (10.7)	2.9 (5.6)	0.8 (1.6)	5.2 (4.3)	2.5 (3.2)
Ki-67/CD45	26.8 (27.9)	33.7 (30.9)	26.9 (31.1)	10.3 (11.7)	2.9 (6.0)	5.4 (8.5)	117.4 (168.0)	31.3 (45.9)

^aP= Proliferative, EL= Early luteal, LL= Late luteal, M= Menstrual. IEL= Intra Epithelial Leukocytes

Table 8.2a: Standardised weights assigned to CD45⁺ counts along the first 3 linear discriminant functions.

	Linear Discriminant Functions		
	1	2	3
In the stroma	-0.82	0.53	-0.30
Around the glands	-0.18	-1.03	-0.18
IEL	-0.26	0.16	0.96

Table 8.2b: Standardised weights assigned to CD56⁺ counts along the first 3 linear discriminant functions.

	Linear Discriminant Functions		
	1	2	3
In the stroma	-0.84	-0.39	-0.27
Around the glands	0.36	-0.86	0.31
IEL	0.45	0.31	0.82

IEL= Intra Epithelial Leukocytes

Table 8.2c: Standardised weights assigned to CD3⁺ counts along the first 3 linear discriminant functions.

	Linear Discriminant Functions		
	1	2	3
In the stroma	-0.60	-0.69	-0.34
Around the glands	-0.78	0.58	0.11
IEL	0.08	-0.37	0.91

IEL= Intra Epithelial Leukocytes

Table 8.2d: Standardised weights assigned to ki-67⁺ counts along the first 2 linear discriminant functions.

	Linear Discriminant Functions	
	1	2
Glands	0.99	-0.05
Stroma	-0.12	0.98

Table 8.2e: Standardised weights assigned to the double labelling ki-67⁺/CD45⁺ Counts along the first 3 linear discriminant functions.

	Linear Discriminant Functions		
	1	2	3
Ki-67 (glands)	-0.31	0.90	0.07
Ki-67 (stroma)	-0.12	-0.07	0.06
CD45 (stroma)	0.27	0.25	-0.95
CD45 (around glands)	0.70	0.30	0.39
CD45 (IEL)	-0.13	-0.20	0.48
Ki-67/CD45	0.38	-0.03	0.24

IEL= Intra Epithelial Leukocytes

Chapter Nine

Endometrial Vasculature

Introduction

Progesterone inhibits endothelial cell proliferation ²¹³ and the mitotic activity of the endometrial vasculature ²¹⁴, which may lead to vascular fragility and irregular bleeding patterns.

Morphological changes in the endometrial capillaries may be one of the factors responsible for bleeding. Gaps start to appear between the endothelial cells in the pre-menstrual phase ²¹⁵, the spiral arterioles become larger and longer in the secretory phase of the natural cycle ¹⁸⁷ and the lumina of the subepithelial capillaries increase in diameter in the secretory phase ²¹⁶.

Endometrial blood vessels undergo different morphological changes under the effect of different sex steroids. NET administered orally, in a dose of 300 µg per day, for 2 months led to an increase of the plasmalemmal vesicles of the endothelial cells, reflecting an increase in endothelial permeability ¹⁸⁶. LNG administered through an intra vaginal ring (20µg/day), induced a reduction in the number of endometrial arterioles, with an increase in the endothelial gaps and haemostatic plugs ²¹⁷. Given orally, LNG (30µg/day) induced a similar reduction in the number of spiral arterioles and an increase in the number of dilated subepithelial venules ²¹⁷, while, subdermal LNG implants (Norplant) induced an increase in vascular density ²¹⁸.

There is a controversy in the literature regarding the endometrial vascular density in the natural cycle. Authors reported no variation in the vascular density with different phases of the natural cycle, and concluded that there was no correlation between vascularity and

stromal development ^{218 219 220}. Ota et al. (1998), on the other hand found significant increase in the vascular surface area, diameter, and total number of capillaries in the secretory phase compared to the proliferative phase ²²¹.

CD34 is a glycoprotein expressed on haematopoietic progenitor cells, in the basement membrane of the vascular endothelium ²²², and on the luminal surface of the endothelial cells ²²³. I chose to detect the CD34 antigen, although not an exclusive marker of endothelial cells, in preference to von Willebrand factor which gives a weaker staining, or the less specific antigen *Ulex europaeus* ²²².

Aims

To establish the vascular morphometric changes using the endothelial cell marker, CD34 in the endometrium of the **a.** natural cycle, **b.** in response to trimegestone-based HRT in a dose ranging study, and **c.** to compare the changes in vascular morphometry between 2 sequential HRT regimens; trimegestone-based and the widely used, NET-based.

Women and method

Endometrium of the natural cycle:

The characteristics of the control population (n = 30) are described on page 53. The histological diagnoses, were proliferative (n= 7), early luteal (n= 9), late luteal (n= 7) and menstrual (n= 7).

HRT treated endometrial samples: This study involved women on one of 2 progestogen regimens:

a. One hundred and seventy six postmenopausal women (page 51-52) were given oral trimegestone (0.05, 0.1, 0.25 & 0.5mg per day) from day 15-28, with continuous micronised oestradiol tablets 2mg daily (Hoechst Marion Roussel; Romainville, France, page 90).

Endometrial biopsies were obtained using vabra curette on day 24 of the last treatment cycle.

b. Twenty five postmenopausal women 48-80 (Mean \pm SD, 62.9 \pm 9.3) who were scheduled for vaginal hysterectomy as part of their treatment for uterine prolapse, were given 2 mg of oestradiol valerate daily with NET 1mg/day from day 17 - 28 for 3 cycles (Novartis, Surrey, UK).

Included in the study were healthy women aged over 50 years with intact uterus who were at least six months postmenopausal. The exclusion criteria and the safety parameters were as in group a. The tissue specimens were obtained from the corpus region, and the hysterectomy was performed between day 11 and 21 of the commencement of NET phase of the third treatment cycle.

Immunohistochemistry

Immunohistochemistry for CD34 is described on page 57.

Assessment of the endometrial sections

The pattern of the distribution of the positively stained vascular spaces was assessed in the endometrial samples, and then, ten randomly selected fields ¹⁶⁸ per slide (x200) were captured (page 63), to evaluate the total vascular space area, diameter and number per field. All fields examined were restricted to the functionalis layer. All the vascular spaces positive for CD34 were measured, and that included the collapsed blood vessels since they were lined by CD34⁺ endothelium. The actual measurements of the vascular diameter included the distance spanned from the outer edge of the CD34 positive membrane of one side of the vessel to the outer edge of the CD34 positive membrane of the other side. The measurements were averages of the maximum and minimum diameter.

Images were captured using Axioplan microscope (Carl Zeiss, Herts UK), and a colour video camera (Sony CCD/RGB). The corresponding areas of an image captured with x200 was 0.121mm² using a measurement graticule, and therefore the data presented are per unit area. The measurements were evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, UK).

Statistics

The data did not fulfil the assumptions necessary for using the analysis of variance and t-test, therefore the non-parametric Kruskal-Wallis and Mann-Whitney tests were used.

Results (Table 9)

In the trimegestone- based HRT group, 176 women were randomised to one of the 4 dose groups and 131 completed the study. Their demographic data are as described on page 126.

The total number of the endometrial samples obtained at the end of the study was 129, as 2 women declined to have a biopsy at the end of the study. Endometrial biopsies of women who bled before the day of the biopsy were excluded (n = 12, page 142), and 10 other samples were too scanty for meaningful analysis. One hundred and seven endometrial biopsies were evaluable for morphometric assessment with anti-CD34 antibody (n = 23, 28, 29 & 27 in the 0.05mg, 0.1mg, 0.25mg & 0.5mg trimegestone dose groups, respectively, Figure 9.1).

In the NET-based HRT, 25 women completed 3 months treatment and all had regular withdrawal bleeding prior to their scheduled hysterectomy.

The endometrium of the natural cycle (figure 9.2a)

There was significant difference in the mean % vascular space area between the phases ($P = 0.007$), with smaller area in the early luteal phase. The mean % vascular space area was significantly lower in the early luteal phase compared to the late luteal and menstrual phases of the natural cycle ($P = 0.009$ & $P = 0.001$, respectively, table 9, figure 9.3a). The average diameter of the vascular space was lower in the early

luteal phase compared to the late luteal and menstrual phases ($P = 0.03$ & $P = 0.009$, respectively, table 9, figure 9.3b), while there was a higher mean number of vascular spaces in the menstrual phase, but was not statistically significant in comparison to the other phases of the natural cycle (figure 9.3c).

Trimegestone-treated endometrium (figure 9.2b)

The mean % total vascular space area and average vascular diameter were higher in the high dose group (0.5 mg), however there was no statistically significant difference between the 4 dose groups (figures 9.3a & b). There was a lower mean number of vascular spaces the higher the dose of trimegestone, but this trend was not statistically significant (figure 9.3c).

There was no difference in the vascular parameters studied between women who bled on the day of the biopsy compared to those who had not bled by then (data not shown). There was no evidence of a difference in the endometrial vascularity between the trimegestone treated endometrium and the natural cycle.

Norethisterone-treated endometrium (figure 9.2c)

The mean % vascular space area was higher in the NET group, but significant only in comparison to the natural cycle ($P = 0.03$, figure 9.3a).

The average diameter of the vascular spaces was lower in the NET group and there was significant difference between the NET-treated endometrium compared to the trimegestone-treated endometrium or

the natural cycle ($P = 0.003$, $P = 0.004$, respectively, table 9, figure 9.3b). The mean number of the vascular spaces was higher in the NET group compared to the trimegestone-treated endometrium or the natural cycle ($P = 0.000$, $P = 0.000$, respectively, table 9, figure 9.3c). There was no evidence of apparent difference in the vascular parameters studied between women who had bled by the day of the biopsy and those who had not (data not shown). The timing of the uterine specimen in relation to NET administration had no effect on the endometrial vascularity.

Summary of the study

Endometrial microvasculature was assessed in postmenopausal women who were given trimegestone-based HRT for 6 cycles, or NET-based HRT for 3 cycles compared to the endometrium of the natural cycle. The endometrial biopsies were taken using the vabra curette in women on trimegestone-based HRT, while it was taken from area B of the uterine fundus (Page 76) during hysterectomy in women on NET-based HRT. Immunohistochemistry using anti- CD34 antibody was utilised.

Endometrial vascular space area and diameter were significantly smaller in the early luteal phase compared to the late luteal or menstrual phases. There was no difference in the vascular parameters studied in the 4 dose groups of trimegestone, while NET-based HRT was associated with a significantly higher number of a smaller vascular spaces than in the trimegestone-treated endometrium or that of the natural cycle.

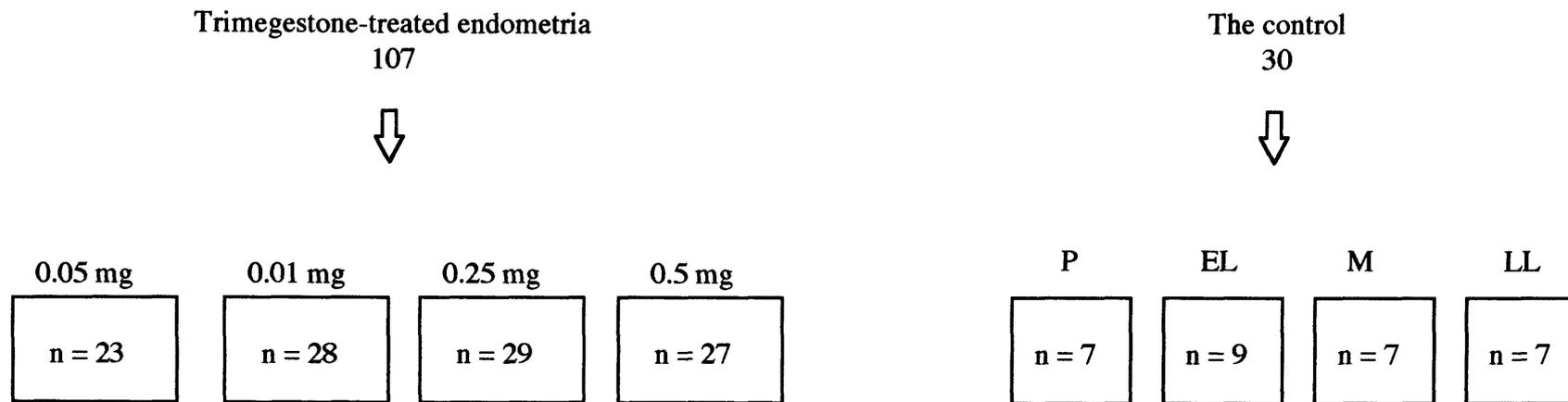
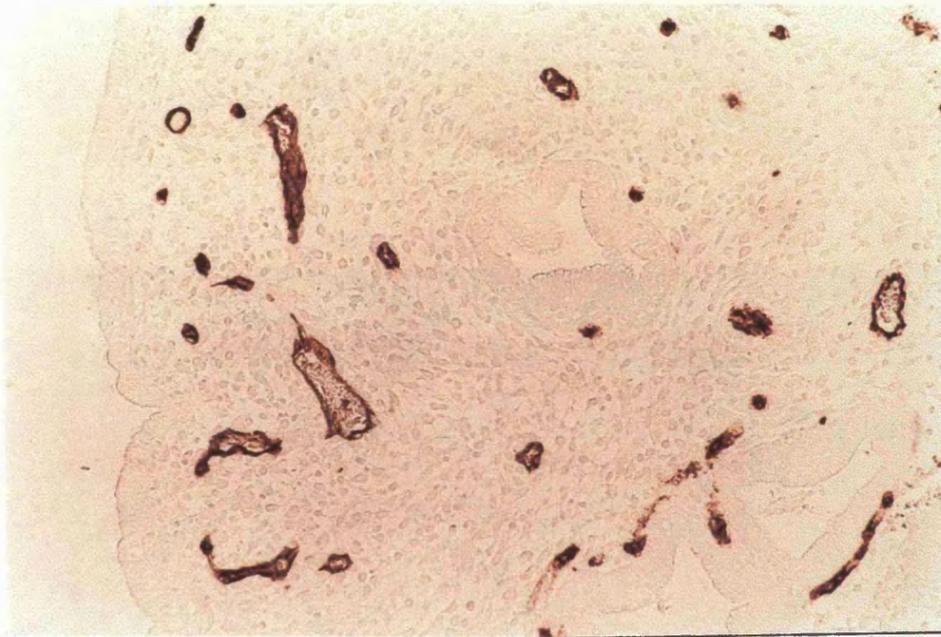
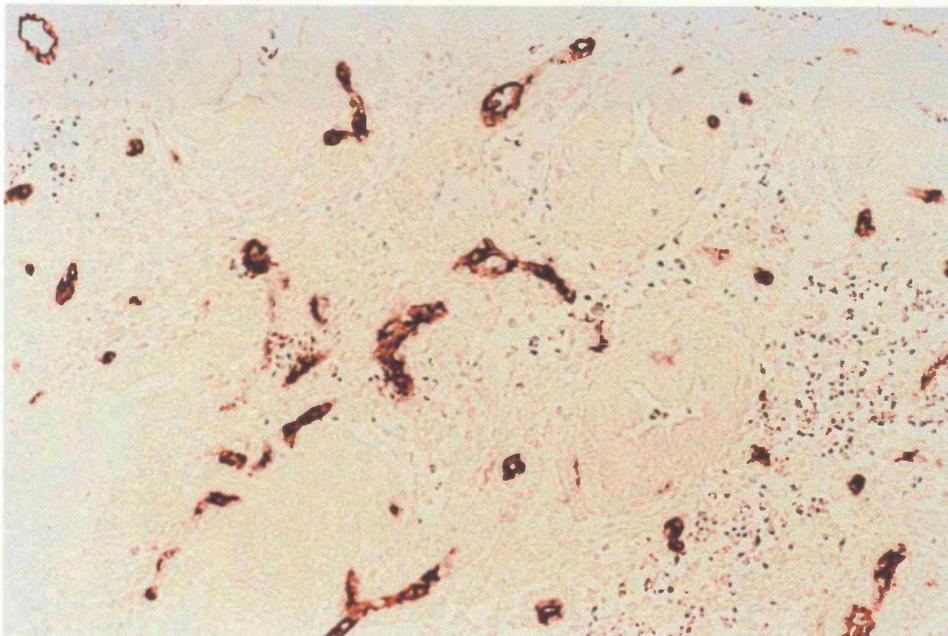


Figure 9.1. Number of specimens in the Endometrial Vascularity study. Total number of specimens are 107, as women who bled before the day of the biopsy were excluded (n = 12), and 10 specimen were too scanty for evaluation. P = proliferative, EL = early luteal, LL = late luteal & M = menstrual.

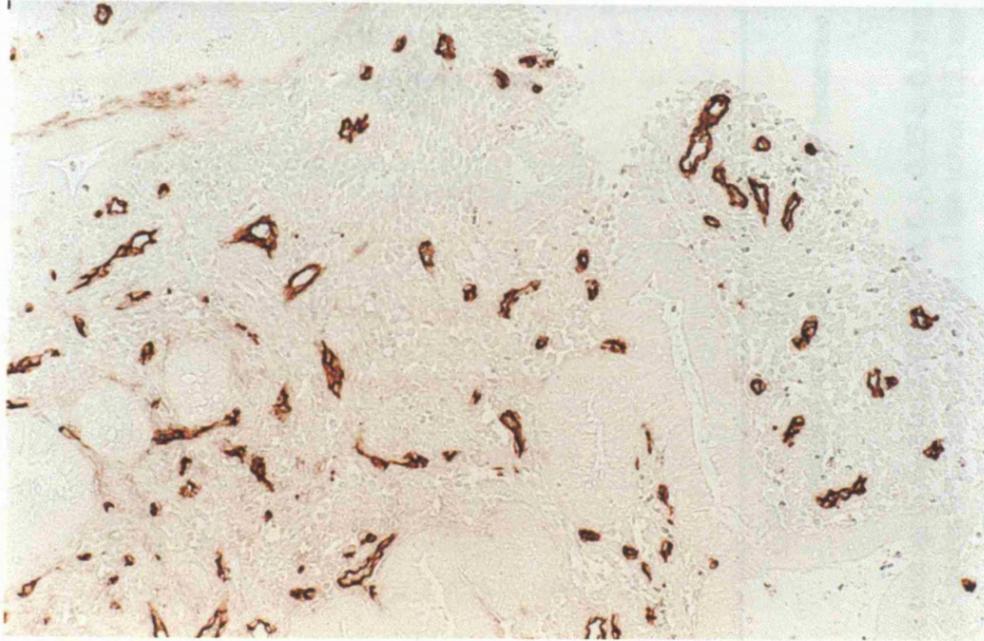


9.2a



9.2b

Figure 9. 2a & 2b: Immunohistochemical staining of endometrial sections of the proliferative phase showing the distribution of CD34⁺ cells in the natural cycle (2a), and trimegestone-based HRT (2b). Original magnification x 200, 1cm = 100µm..



9.2c

Figure 9. 2c : Immunohistochemical staining of endometrial sections, showing the distribution of CD34⁺ cells in the NET-based HRT, original magnification x 200, 1cm = 100 μ m.

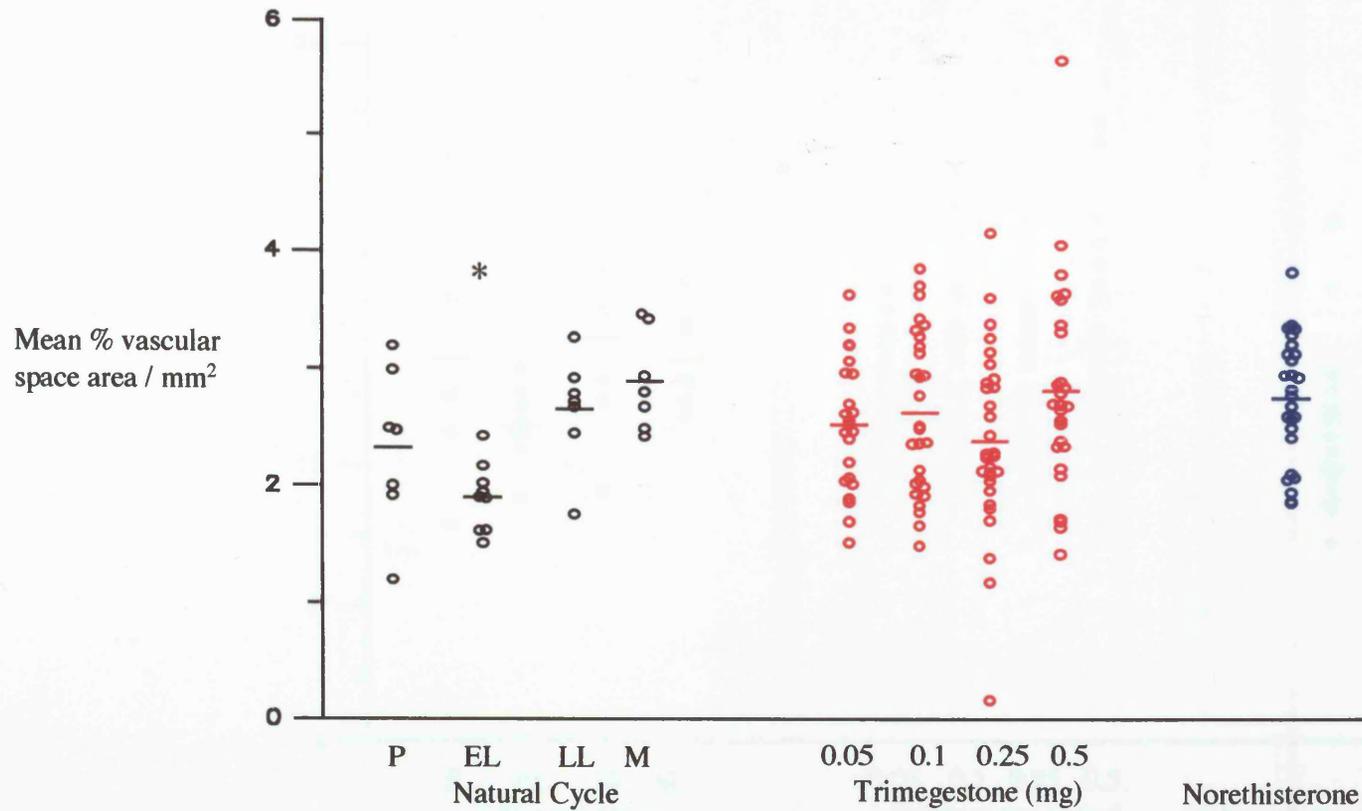


Figure 9.3a: The mean % vascular space area in the 4 dose groups of trimegestone-based HRT (0.05, 0.1, 0.25 & 0.5 mg), the norethisterone-based HRT and the 4 phases of the natural cycle, Proliferative (P), Early luteal (EL), Late luteal (LL) and Menstrual (M). Open circles are used for the mean % vascular space area in the individual woman. * The % vascular space area is significantly lower in the early luteal phase compared to the late luteal and menstrual phases ($P=0.009$, $P = 0.001$, respectively).

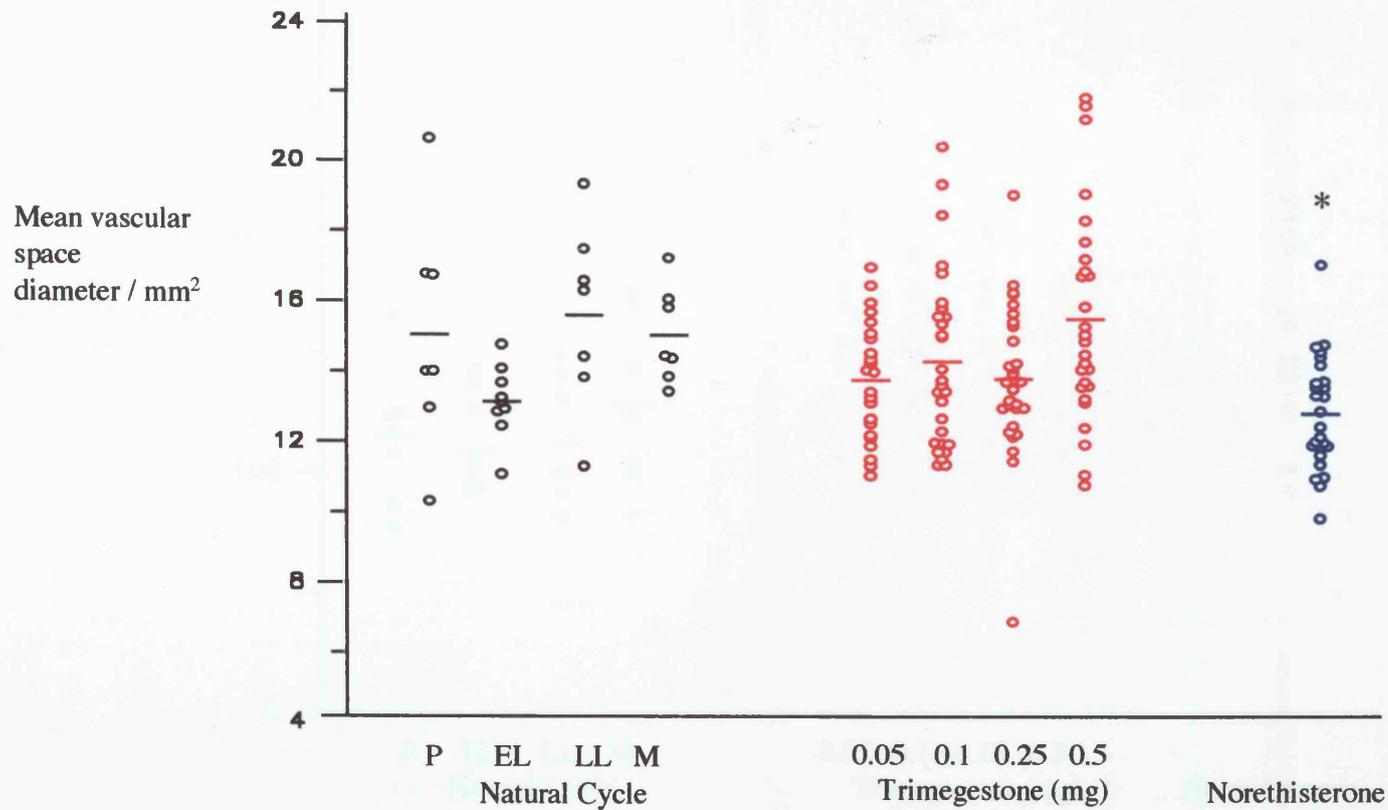


Figure 9.3b: The mean vascular space diameter in the 4 dose groups of trimegestone-based HRT (0.05, 0.1, 0.25 & 0.5 mg), the norethisterone-based HRT and the 4 phases of the natural cycle, Proliferative (P), Early luteal (EL), Late luteal (LL) and Menstrual (M). Open circles are used for the mean diameter of the vascular space in the individual women. * There mean vascular space diameter was smaller in the NET group compared to the trimegestone-treated endometrium or that of the natural cycle ($P = 0.003$, $P = 0.004$, respectively).

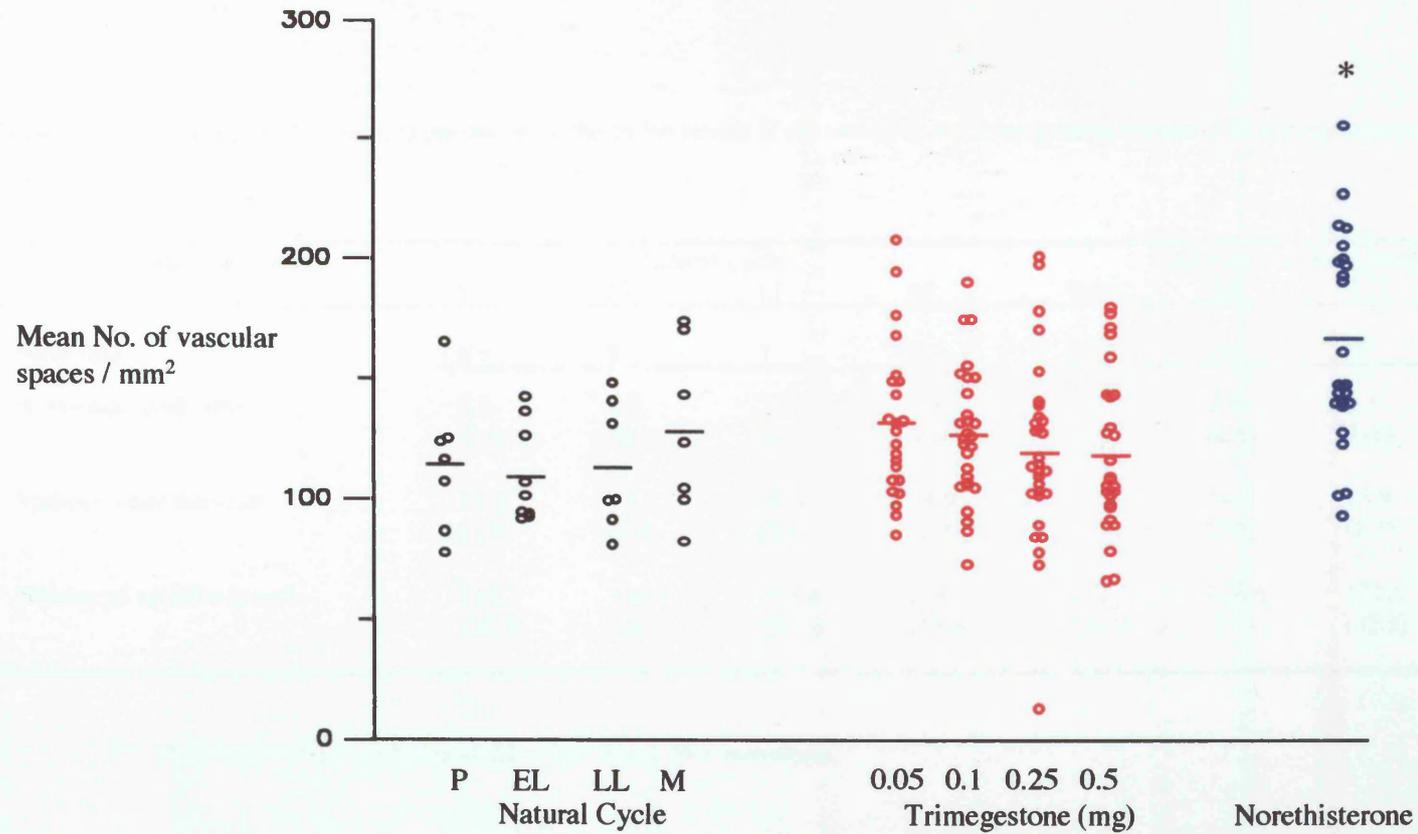


Figure 9.3c: The mean number of the vascular spaces in the 4 dose groups of trimegestone-based HRT (0.05, 0.1, 0.25 & 0.5 mg), the norethisterone-based HRT and the 4 phases of the natural cycle, Proliferative (P), Early luteal (EL), Late luteal (LL) and Menstrual (M). Open circles are used for the mean number of the vascular spaces in the individual woman. * The mean number of the vascular spaces was higher in the NET-group compared to the trimegestone-treated endometrium (P = 0.000) or that of the natural cycle (P = 0.000).

Table 9 : Mean and (SD) of the vascular parameters in the endometrium of the natural cycle, Trimegestone-treated or Norethisterone-treated (NET). Data are per mm² endometrial tissue.

Vascular parameters	P	Natural cycle			Trimegestone dose group (mg)				NET
		EL	LL	M	0.05	0.1	0.25	0.5	
Mean (SD)	n = 7	9	7	7	23	28	29	27	22
% vascular space area	2.3 (0.6)	1.9 (0.2)	2.5 (0.4)	2.8 (0.4)	2.4 (0.5)	2.5 (0.6)	2.3 (0.6)	2.7 (0.8)	2.7 (0.5)
Vascular space diameter	15.0 (3.3)	13.1 (1.0)	15.4 (2.6)	14.9 (1.3)	13.7 (1.7)	14.2 (2.5)	13.9 (1.7)	15.4 (3.0)	12.9 (1.6)
Number of vascular spaces	114.7 (28.7)	110.8 (20.1)	113.4 (26.6)	128.6 (35.6)	131.5 (32.1)	126.6 (27.9)	122.5 (32.9)	117.8 (32.9)	166.9 (46.8)

P = proliferative, EL = early luteal, LL = late luteal, M = menstrual.

Chapter Ten
Apoptosis

Introduction

Apoptosis is an active process of cell death, and is as important as proliferation for the maintenance of tissue architecture. Apoptosis develops in 2 stages: first it is characterised by nuclear condensation, pyknosis, cell shrinkage and nuclear fragmentation; together with the formation of small ovoid cytoplasmic fragments containing remnants of nuclei, the apoptotic bodies ²²⁴. DNA laddering was observed in apoptosis suggestive of definable points of genomic breakage, while extensive smearing was noted in necrosis ²²⁵. The second stage involves the rapid disappearance of apoptotic cells from the tissue through phagocytosis, which are degraded within 1-2 hours, thus making the histological assessment of the extent of apoptosis difficult ²²⁶. This rapid nature of apoptosis means that even a small number of apoptotic cells in the tissue reflect a considerable cell turnover.

The process of apoptosis in the endometrium is not fully understood, since it involves the activation of many pro-apoptotic and anti-apoptotic genes. Bcl-2 is a proto-oncogene whose protein prolongs cell survival by preventing apoptosis. Bcl-2 is localised in the outer and inner mitochondrial membranes, the nuclear membrane and the endoplasmic reticulum ^{227 228 229}.

Bax, is a pro-apoptotic protein and a member of the bcl-2 family, with a 21% amino acid sequence homology with bcl-2 ¹⁶⁴. Most members of the bcl-2 family possess bcl-2 homology regions (BH), which determine

their ability to interact with each other, where they homo- and heterodimerize through these BH domains ^{230 231}. The ratio of bcl-2 to bax within the cell determines the susceptibility of that cell for apoptosis.

In the ischaemia-reperfusion injury model as described by Markee (1940) ⁹⁸, intracellular Ca^{+2} is released, which in endometrial explants can serve to illustrate an initiating events for cell damage and tissue disintegration at menstruation. Cell damage occur consequent upon mitochondrial membrane permeabilisation resulting in cytochrome c release, which promotes a sequence of events leading to the formation of caspase-9 and caspase-3 (CCP32) activation complex – the apoptosome. This leads to the cleavage of different intracellular proteins, the disruption of cellular architecture, and finally apoptosis ¹⁶¹

^{162 163}

The caspases are cysteine proteases that are activated in cascade fashion leading to cell death ²³². Krajewska et al. (1997) reported a cytoplasmic expression of caspase-3 in epithelial cells at the luminal apex of the endometrial cells ¹⁶⁷.

The detection of ki-67 antigen expression by immunohistochemical technique is used to assess proliferation in preference to PCNA, which is expressed in all phases of the cell cycle ²³³.

Aims

The aims of this study were **a.** To assess the distribution of the apoptotic bodies, bcl-2, bax, caspase-3 and ki-67 in the endometrium in different phases of the natural cycle. **b.** To assess the distribution of these apoptotic and anti-apoptotic markers in the trimegestone-based HRT. **c.** To consider whether any of the markers distinguishes between the phases of the natural cycle and the 4 dose of trimegestone. **d.** To consider whether the expression of any of these markers was dependent on the anticipated onset of the progesterone associated bleeding in the trimegestone-treated women.

Women and Methods

a. The control

The characteristics of the endometrial samples obtained during the natural cycle are described on page 53. The histological diagnoses, were proliferative (n= 9), early luteal (n= 10), late luteal (n= 10) and menstrual (n= 8).

b. Women treated with trimegestone-based HRT

Postmenopausal women (n = 176, page 51-52) were given oral trimegestone (0.05, 0.1, 0.25 & 0.5mg per day) from day 15-28 with continuous micronised oestradiol 2mg daily (Hoechst Marion Roussel; Romainville, France), for 6 treatment cycles as described on page 90. Endometrial biopsies were obtained on day 24 of the last treatment cycle. The endometrial samples were fixed immediately in 10% formal-

saline embedded in paraffin and 5 µm sections were stained with H&E for histological assessment of the of the phase of the natural cycle (standard protocol at histopathology department, Leicester Royal Infirmary).

Immunohistochemistry

The immunohistochemical techniques used for the detection of bcl-2, bax, caspase-3 and ki-67 are described on page 55.

Assessment of the endometrial sections

The overall patterns of the distribution of the positively stained cells were described in the endometrial slides, and then fifteen randomly selected fields ¹⁶⁸ per slide (x200) were captured (Page 63), to evaluate the total glandular area, percent of glandular staining (for bcl-2, bax and caspase-3), the number of positively stained glandular cells (for ki-67) or the positive stromal cells. To assess the number of bcl-2 or bax positively stained stromal cells per field, fifteen randomly selected fields ¹⁶⁸ per slide were captured (x1000) under oil immersion. All evaluated fields were restricted to the functionalis layer.

Images were captured using Axioplan microscope (Carl Zeiss, Herts UK), and a colour video camera (Sony CCD/RGB). The data presented are per unit area and the corresponding areas of an image captured with x200 and x1000 were 0.121mm² and 0.0046 mm² , respectively using a measurement graticule. The areas measured or cell counted

were evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, UK).

Statistics

The Kruskal-Wallis test was used to make an overall comparisons of the markers in the different phases of the natural cycle and the different doses of trimegestone. Specific pairs of phases or doses were compared using the Mann-Whitney test. A linear discriminant analysis¹⁹³ was used to investigate whether weighted contributions of the markers can distinguish between the eight groups (4 phases of the natural cycle, and 4 doses of trimegestone).

Results

In the MRU, Leicester, 176 women were randomised to one of the 4 dose groups and 131 completed the study, their demographic data are as described in page 126.

Endometrial biopsies of women who bled before the day of the biopsy were excluded (n = 12, page 142), 11 other samples were too scanty for meaningful analysis, and 106 endometrial biopsies were evaluable for morphometric assessment, (24, 28, 27 & 27 in the 0.05mg, 0.1mg, 0.25mg & 0.5mg trimegestone dose groups, respectively. Figure 10.1).

The endometrium of the natural cycle (Table 10.1)

There was no discernible difference in the expression of bax and ki-67 between the functionalis and the basalis layers of the endometrium. Bcl-2, however, was more prevalent in the endometrial glands and stroma of the basalis compared to the functionalis layer. The density of bcl-2⁺ cells in the stroma was much lower than that for bax⁺ cells. There was a tendency for periglandular aggregation of bcl-2⁺ and caspase-3⁺ cells. Endothelial cells were positive for bcl-2 and bax, but not for ki-67 or caspase-3.

Apoptotic bodies

Examination of sections stained with H&E showed no difference in the prevalence of the apoptotic bodies in the glands of different phases of the natural cycle. In the stroma, apoptotic bodies were very infrequently identified and therefore not included in further analysis (Figure 10.2a).

Bcl-2

Bcl-2 was expressed in the cytoplasm of glandular epithelium and stromal cells (Figure 10.2b & 2c). There was a significantly higher staining in the glands in the proliferative phase compared to the other phases of the natural cycle ($P < 0.003$). In the stroma, there was gradual increase in bcl-2⁺ cells in the early and late luteal phases, and was highest in the menstrual phase, but this trend was not statistically significant (Figure 10.3a & 10.3b).

Bax

Bax immunostaining was expressed diffusely in the cytoplasm of glandular epithelium, but showed a peri-nuclear distribution in the stromal cells (Figure 10.2d & 2e). The expression of Bax in the glands showed no variation across the phases of the natural cycle, while in the stroma, it increased gradually from the proliferative phases of the natural cycle, and was highest in the late luteal phase before it declined in the menstrual phase.

Caspase-3

Glandular epithelium expressed caspase-3 in the cytoplasm (Figure 10.2f). In the stroma, caspase-3 was expressed mainly in the nucleus with the tendency for the caspase-3⁺ cells to group around the glands (Figure 10.2g).

Caspase-3 expression in the glandular epithelium decreased gradually across the phases of the natural cycle, and it was lowest in the menstrual phase. There was a statistically significantly higher expression in the proliferative phase compared to the late luteal phase ($P < 0.04$). In the stroma, there was a low expression of caspase-3 in the proliferative and early luteal phase, but increased significantly in the late luteal and the menstrual phases ($P < 0.02$ and $P < 0.05$, respectively, Figure 10.3c & 10.3d).

Ki-67

Ki-67⁺ cells were present in the endometrial glands but more frequently expressed in the stroma (Figure 10.2h & 2i). The ki-67 antigen expression in the glands was significantly higher in the proliferative and early luteal phases compared to other phases of the natural cycle ($P < 0.0006$ and $P < 0.004$, respectively; Figure 10.3e & 3f). In the stroma the ki-67 expression remains relatively constant during the different phases of the natural cycle.

The trimegestone-treated endometrium (Table 10.1)

The stromal density of bcl-2⁺ cells was much lower than that for bax⁺ cells, in a profile similar to the endometrium of the natural cycle.

There was a tendency for periglandular aggregation of caspase-3⁺ and bcl-2⁺ cells. Endothelial cells were positive for bax and bcl-2, but not for caspase-3 or ki-67.

There was no dose response effect on the endometrial expression of the apoptotic bodies, bax⁺, caspase-3⁺ or ki-67⁺ cells, or the glandular epithelial expression of bcl-2⁺ cells. Bcl-2 expression, however, was significantly lower in the stroma in the 0.25mg dose compared to the 0.1 mg dose group ($P = 0.005$).

Correlation between the expression of different markers in endometrial samples

The endometrium of the natural cycle: There was statistically significant positive correlation between bcl-2⁺ stromal cells and caspase-3⁺ glandular epithelial cells in the late luteal phase ($P < 0.01$). Similarly there was a positive correlation between the number of caspase-3⁺ stromal cells and ki-67⁺ stromal cells in the late luteal phases ($P = 0.001$, Table 10.2).

Trimegestone-treated endometrium: In the 0.05 mg trimegestone dose group, there were positive correlations between bcl-2⁺ stromal cells and bcl-2⁺ glandular epithelial cells, and ki-67⁺ stromal cells and bcl-2⁺ glandular epithelial cells, and bcl-2⁺ stromal cells and ki-67⁺ stromal cells ($P < 0.01$). In the 0.1 mg dose group there was a positive correlation between the glandular epithelial apoptotic bodies identified in the H&E sections and bcl-2⁺ stromal cells, and a similar correlation between caspase-3 expression in the stroma and the glands ($P < 0.01$).

Effect of the expected day of onset of progestogen associated bleeding (PAB)

The endometrial samples in the trimegestone-treated groups were collected at a fixed point of the treatment cycle i.e. day 10 of the trimegestone administration. Given the fact that these women experienced different mean day of onset of bleeding (chapter 4), it was reasonable to assume that endometrial development may have been

different in these samples due to factors other than the dose or duration of the progestogen administered. The prediction of the day of onset of bleeding in the following cycle in the trimegestone-treated groups was explained in chapter 4. I correlated the endometrial expression of these markers with the expected day of onset of bleeding of each woman in the study, and found that the later the expected day of onset of bleeding the lower was the expression of the apoptotic bodies ($P = 0.002$, Table 10.3a). This finding was consistent even when individual trimegestone dose groups were examined.

However, when I restricted the analysis to include only women with regular cycle length ($SD \leq 1$ day), there appeared to be a negative correlation between the expected day of onset of bleeding and $bcl-2^+$ - and bax^+ - stromal cells ($P = 0.021$, $P = 0.016$ respectively, Table 10.3b). This correlation was not maintained when individual dose groups were examined, possibly due to the lower numbers remaining in each dose group.

Linear discriminant analysis

There was correlation between the different apoptotic markers, however there was no dose response effect on the expression of these markers. Linear discriminant analysis was utilised to look at best parameters that distinguish between the 4 doses of trimegestone and 4 phases of the natural cycle. The trimegestone-treated endometria had similar expression of apoptotic markers, regardless of the dose (figure 10.4). The first linear discriminant function separates the trimegestone

group from the proliferative and early luteal phases, while the second linear discriminant separates the proliferative from the early luteal phase. The first linear discriminant function was dominated by ki-67⁺ glandular epithelial cells, which had a higher discriminant value, i.e. the proliferative and early luteal phases had a higher expression of ki-67 in the glands than trimegestone or the other phases of the natural cycle. The second linear discriminant function depended on apoptotic bodies identified in the H&E sections, bcl-2⁺ glandular epithelial cells and caspase-3⁺ stromal cells. These markers were expressed more in the menstrual and late luteal phases than in the trimegestone treated endometria. The LDA did not show any contribution of the studied markers that distinguish between the doses of trimegestone (Table 10.4).

Summary of the study

In this study, I have described the immunohistochemical expression of pro-apoptotic and anti-apoptotic markers in the endometrium of the natural cycle and that of trimegestone-based HRT given to postmenopausal women for 6 cycles. Apoptotic bodies were evaluated in H&E stained sections. Ki-67 immunopositive cells were evaluated as indicators of cellular proliferation in endometrial sections.

There was no difference in the number of apoptotic bodies in the endometrial glands of the natural cycle. Bcl-2 and caspase-3 expression in the glandular epithelium was significantly higher in the proliferative phase compared to the other phases of the natural cycle.

In the stroma, bcl-2 expression increased as the endometrium progressed from the proliferative phase of the natural cycle and was highest in the menstrual phase, while caspase-3 expression was significantly higher in the late luteal and menstrual phases.

Bax expression in the stroma increased in late luteal phase, while ki-67 was significantly higher in the glands in the proliferative and early luteal phases.

Apart from lower expression of bcl-2 in the stroma in dose 0.25 mg, there was no significant dose-response effect of trimegestone.

Utilising the linear discriminant analysis, the apoptotic markers in the trimegestone-treated endometrium had similar expression, regardless of the dose, and were distinguished from the phases of the natural cycle.

There was weak correlation between the different markers in the endometrium of the natural cycle and that of trimegestone-based HRT.

While accepting the fact in principle that correlation does not imply causation, such association may sometimes be useful in the understanding of a particular phenomenon. Indeed, they may even initiate new concepts.

I assessed the correlation of these markers and the calculated day of onset of PAB. The later the calculated day of onset of the PAB the lower was the glandular apoptotic bodies and stromal count of bcl-2 or bax.

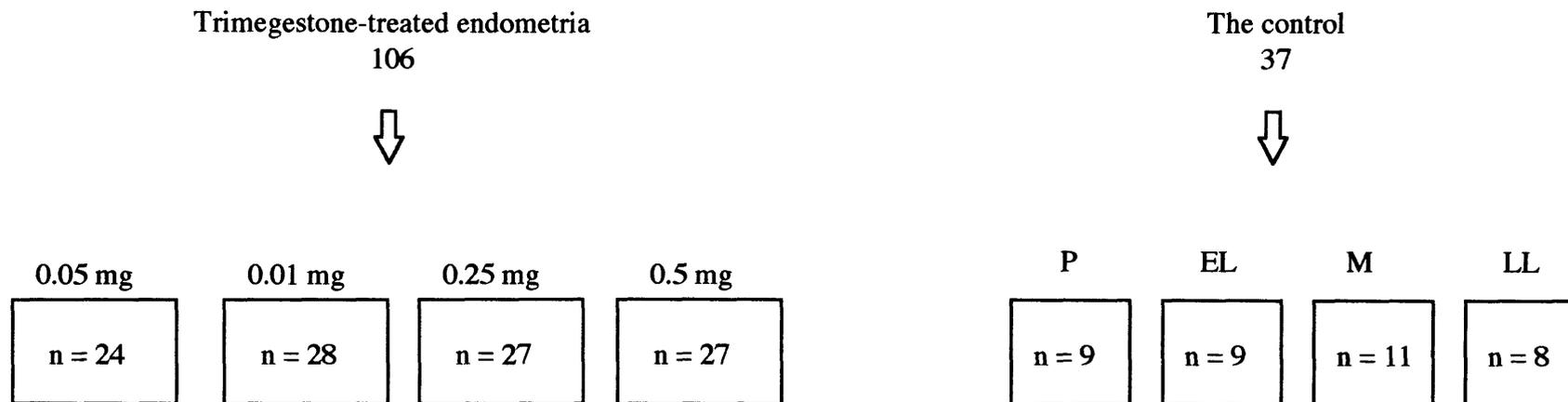
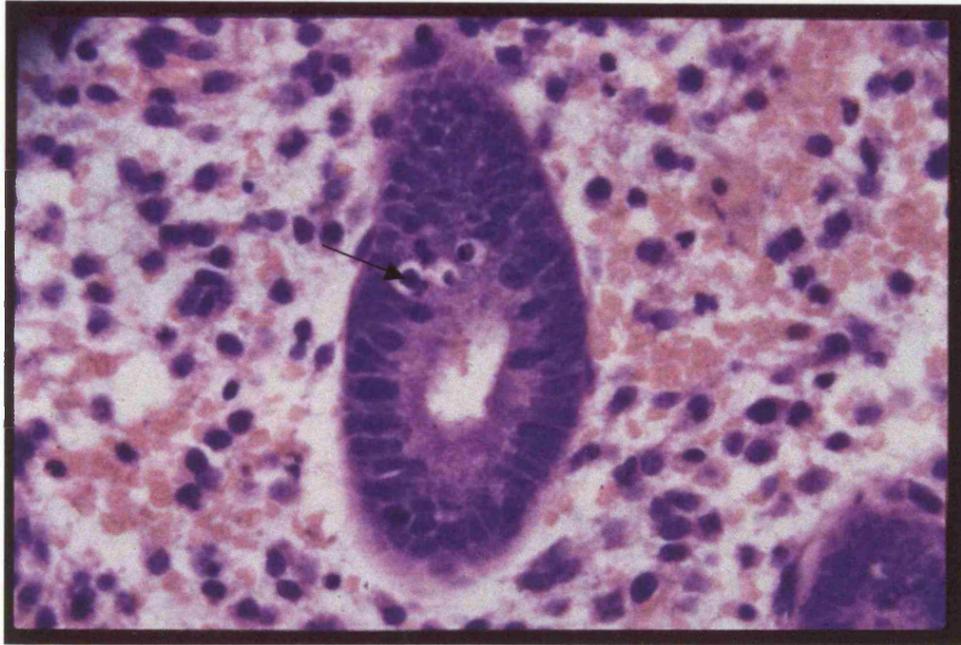
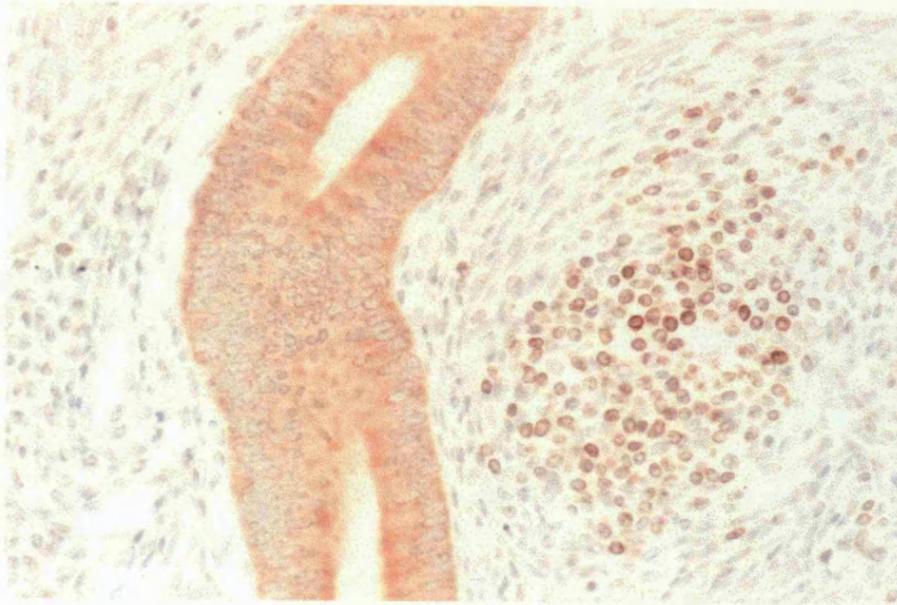


Figure 10.1. Number of specimens in the Apoptosis study. Total number of specimens are 106, as women who bled before the day of the biopsy were excluded, n = 12, and 11 specimens were too scanty for evaluation. P = proliferative, EL = early luteal, LL = late luteal & M = menstrual.

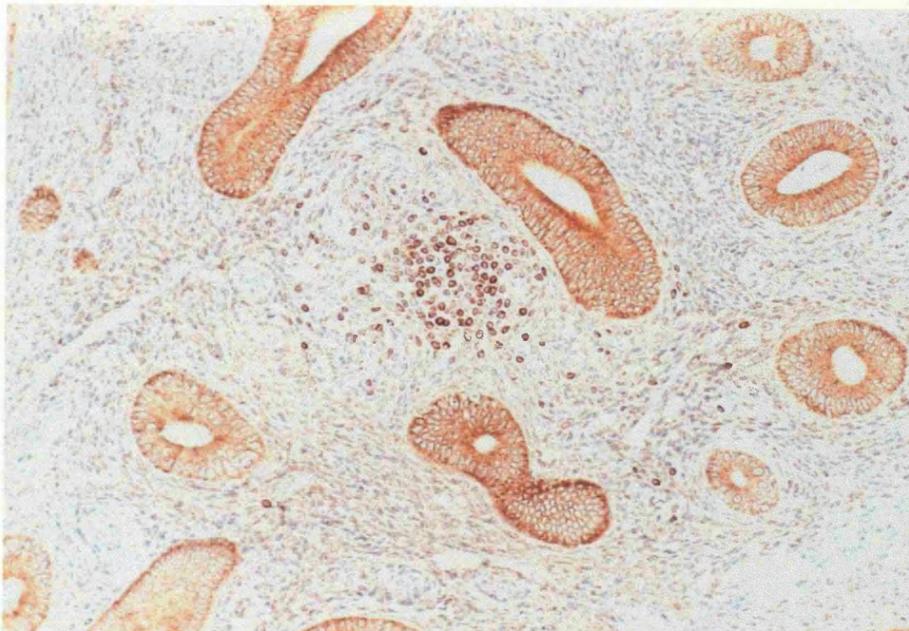


10.2a

Figure 10. 2a. Endometrial section showing apoptotic bodies, arrow (H&E) in the late luteal phase. Original magnification x 1000, 1 cm = 25 μ m.



10.2b

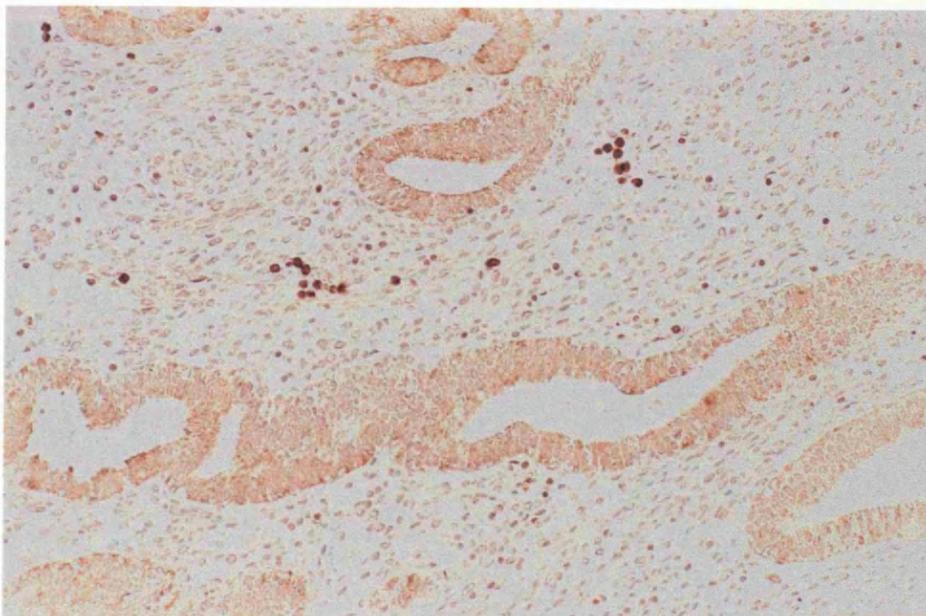


10.2c

Figure 10.2b & 2c: Immunohistochemical staining of endometrial sections in proliferative phase showing periglandular distribution of bcl-2 positive stromal cells around the glands, original magnification x 400, 1cm = 50 μ m, (2b). Immunohistochemical staining showing cytoplasmic staining of the glands and stroma, original magnification x 200, 1cm = 100 μ m (2c).

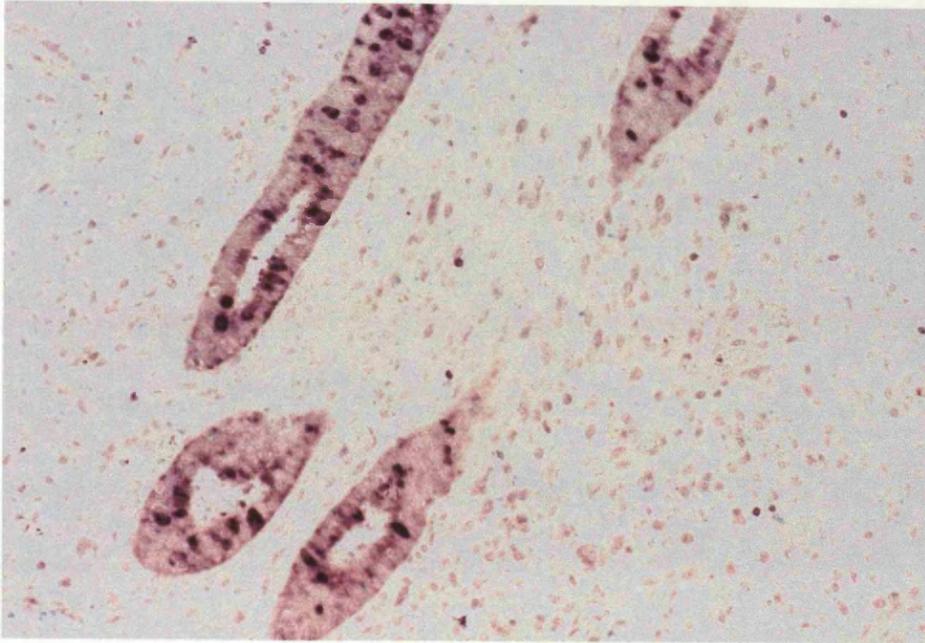


10.2d



10.2e

Figure 10.2d & 2e: Immunohistochemical staining of endometrial sections in the mid luteal phase showing brown staining of the glands, original magnification x 200, 1cm = 100 μ m (2d). Immunohistochemical staining showing brown staining in the stroma and glands in the early luteal phase, original magnification x 200, 1cm = 100 μ m (2e),

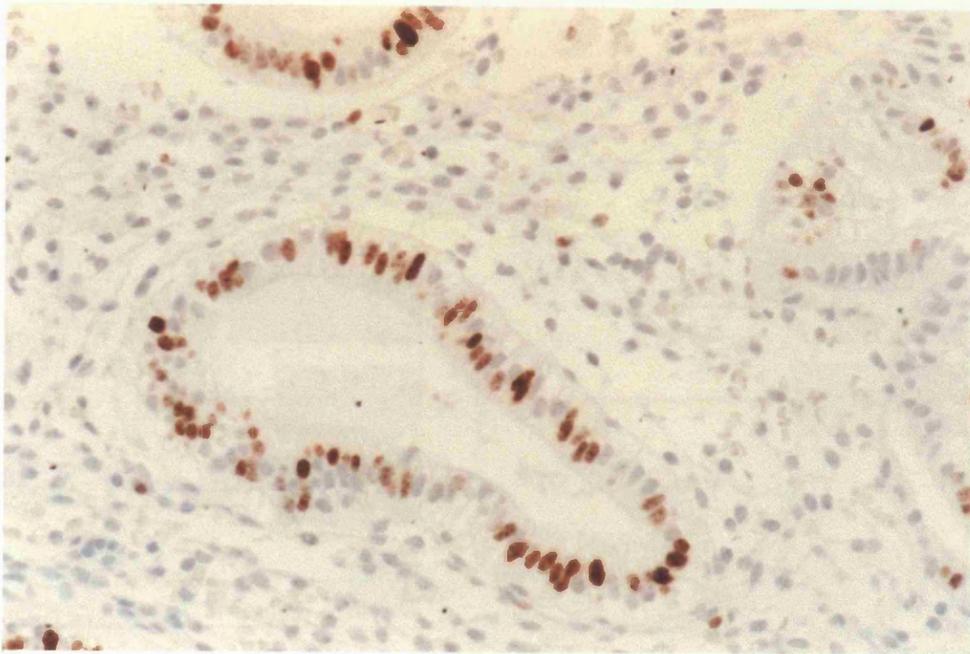


10.2f

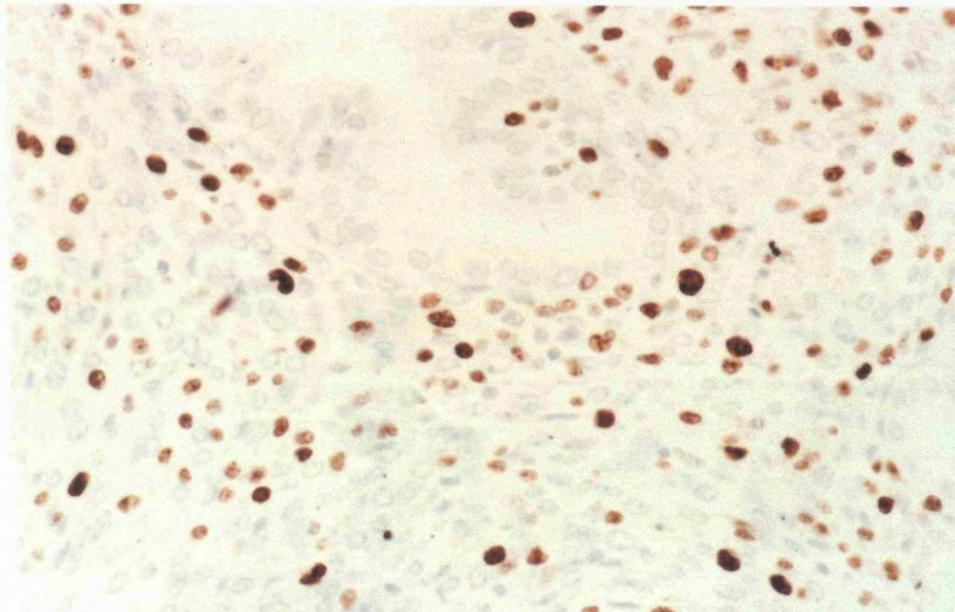


10.2g

Figure 10.2f & 2g: Immunohistochemical staining of endometrial sections in the proliferative phase showing staining of the glands, original magnification x 200, 1cm = 100 μ m (2f). Immunohistochemical staining showing periglandular aggregation of caspase-3⁺ cells in the mid luteal phase, original magnification x 200, 1cm = 100 μ m (2g).

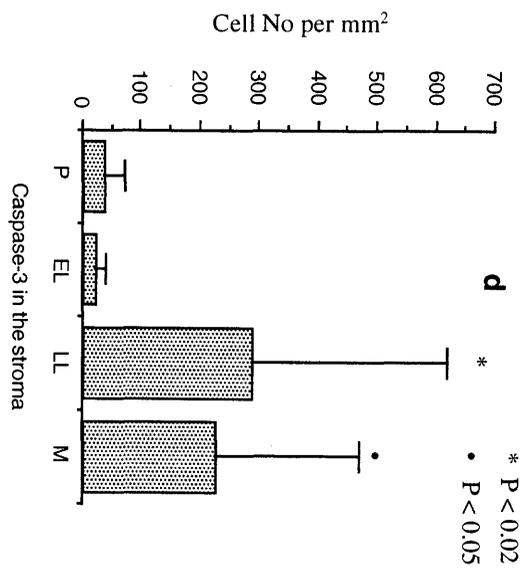
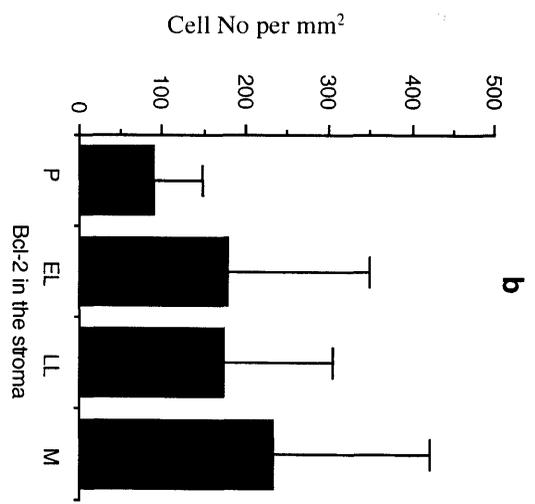
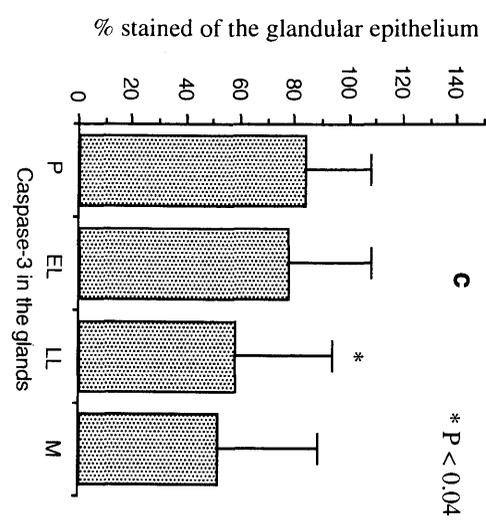
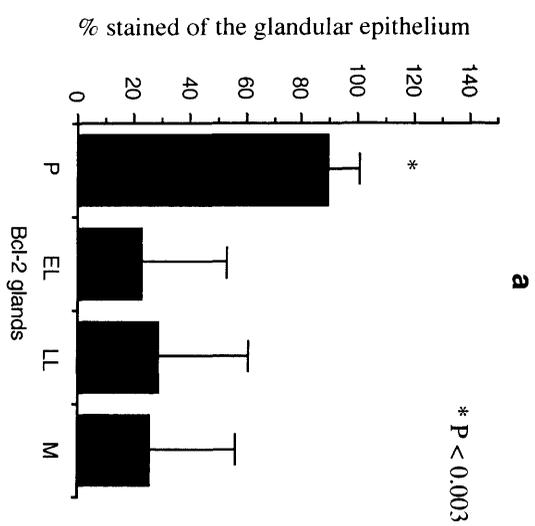


10.2h



10.2i

Figure 10.2h & 2i: Immunohistochemical staining of endometrial sections in the mid luteal phase showing ki-67 nuclear staining of the glands, original magnification x 200, 1cm = 100 μ m (2h). Immunohistochemical staining showing Ki-67 in the stroma in the late luteal phase, original magnification x 200, 1cm = 100 μ m (2i),



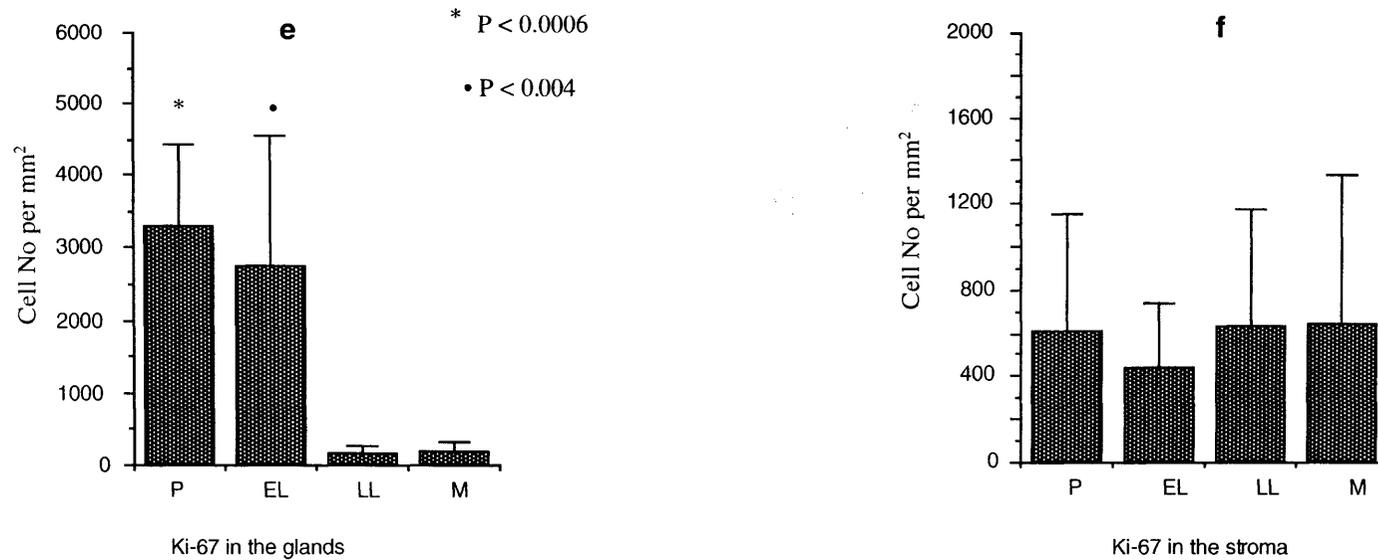


Figure 10.3: **a.** Mean and standard deviation of percent glandular staining with bcl-2. The glandular expression was statistically significantly higher in the proliferative phase (P) compared to the other phases of the natural cycle ($P < 0.003$). **b.** Number Bcl-2⁺ cells in the stroma of the natural cycle. **c.** % glandular staining of caspase-3, which was significantly higher in the proliferative phases compared to the late luteal phase ($P < 0.04$). **d.** Number of caspase-3⁺ stromal cells. The expression was significantly higher in the late luteal (LL) phase compared to the proliferative or early luteal phases (EL, $P < 0.02$), and was significantly higher in the menstrual phase compared to the proliferative or early luteal phases ($P < 0.05$). **e.** Number of ki-67⁺ glandular epithelial cells. The expression was significantly higher in the proliferative phase compared to the late luteal and menstrual phases (M, $P < 0.0006$), while significantly higher in the early luteal phase compared to the late luteal and menstrual phases ($P < 0.004$). **f.** Number of ki-67⁺ stromal cells.

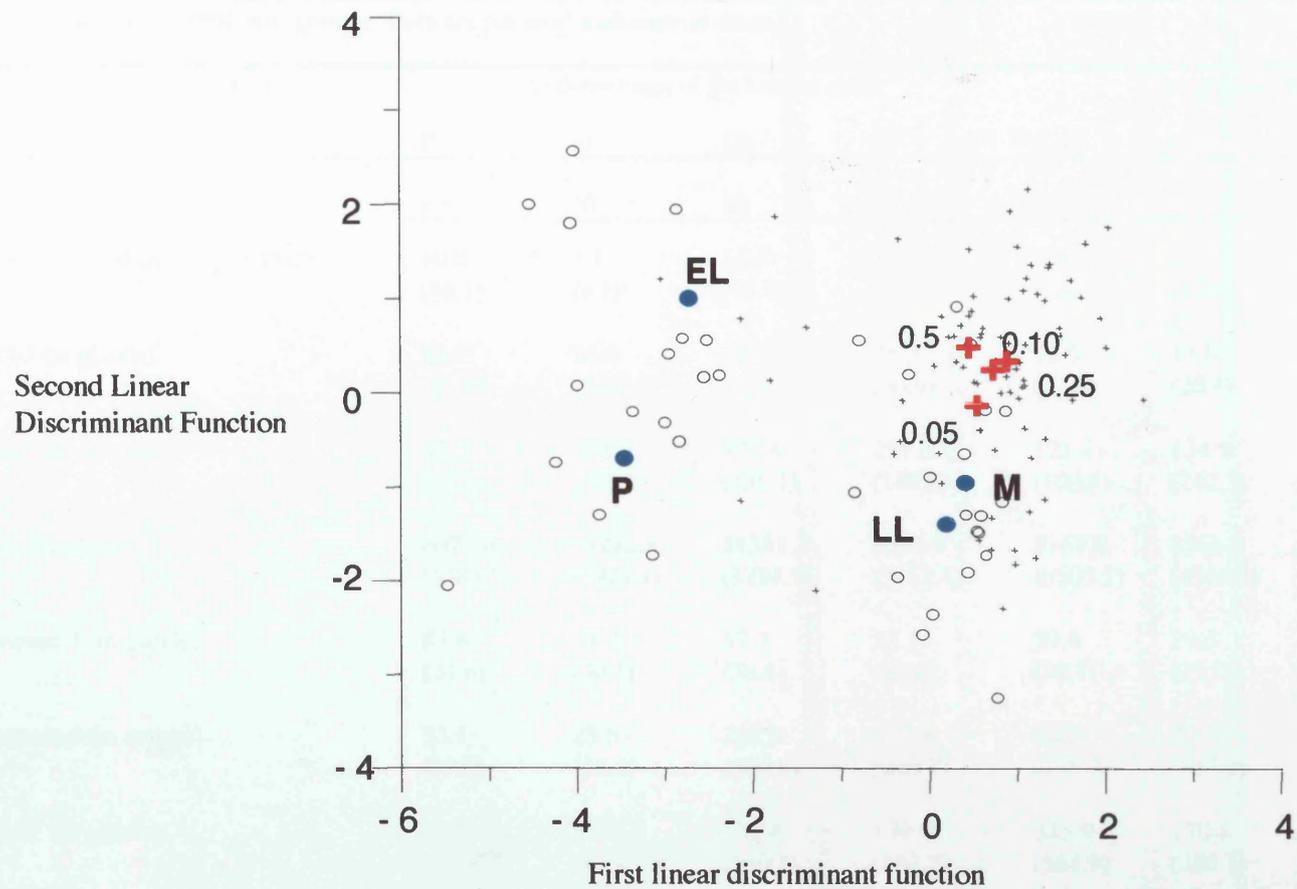


Figure 10.4: The relationship between linear discriminant analysis scores of the apoptotic markers in the trimegestone-treated endometrium and the 4 phases of the natural cycle. Black crosses represent the mean score of the individual sample in the treated group, and the red crosses represent group means. Black circles represent mean score of the individual sample in the control and the green circles represent the group means. P= Proliferative, EL= Early luteal, LL= Late luteal & M= Menstrual.

Table 10.1. Means and standard deviation (SD) of the apoptotic markers in the endometrium of the natural cycle and in the endometrium treated with sequential trimegestone. Data are per mm² endometrial tissue.

Apoptotic markers		Endometrium of the natural cycle ^a				Trimegestone dose groups (mg)			
		P	EL	LL	M	0.05	0.1	0.25	0.5
		n = 9	10	10	8	20	23	27	25
Apoptotic bodies	Mean (SD)	10.8 (16.1)	3.1 (4.1)	11.2 (11.5)	11.8 (16.5)	4.4 (5.0)	2.5 (3.2)	3.8 (9.1)	2.6 (4.4)
Bcl-2 (in glands)*		88.7 (11.9)	22.3 (30.9)	28.8 (32.6)	25.3 (30.9)	15.5 (22.5)	17.0 (20.4)	12.4 (14.9)	18.9 (26.1)
Bcl-2 (in stroma)		87.7 (60.3)	176.7 (172.9)	170.8 (135.1)	231.8 (188.8)	121.3 (100.8)	134.9 (242.3)	94.6 (76.3)	197.7 (215.6)
Bax (in stroma)		8071.6 (5583.5)	11282.8 (2921.9)	11381.3 (3784.7)	8663.4 (3662.4)	8167.8 (6505.5)	8366.2 (4368.8)	9164.3 (4040.2)	7509.7 (3383.6)
caspase-3 (in glands)*		83.4 (24.6)	77.7 (30.5)	57.3 (36.4)	51.1 (37.4)	39.6 (30.7)	29.5 (25.1)	33.0 (27.0)	41.4 (30.5)
caspase-3 (in stroma)		35.1 (39.0)	23.5 (18.4)	288.9 (330.8)	227.8 (240.7)	85.3 (146.9)	70.2 (153.0)	65.6 (93.7)	31.7 (38.9)
Ki-67 (in glands)		3277.0 (1168.5)	2756.4 (1786.4)	135.8 (140.1)	170.6 (162.2)	345.9 (984.9)	170.8 (386.3)	411.7 (978.6)	380.9 (739.4)
Ki-67 (in stroma)		608.0 (543.4)	433.8 (305.9)	626.4 (549.3)	639.7 (696.2)	498.8 (387.1)	633.0 (273.2)	538.0 (330.4)	497.2 (400.0)

^a P = proliferative, EL = Early luteal, LL = Late luteal, M = Menstrual. * = % staining of the glands

Table 10.2. The correlation between the apoptotic markers a. in the different phases of the natural cycle, b. in the 4 doses of trimegestone.
 1 = Apoptotic bodies, 2 = Bax + Stromal cells, 3 = Bcl-2 in the glandular epithelium, 4 = Bcl-2+ Stromal cells, 5 = Caspase-3 in the glandular epithelium, 6 = Caspase-3+ Stromal cells, 7= ki-67 in the glandular epithelium, 8= ki-67+ Stromal cells. Underlined values = P < 0.05

a. The natural cycle
 Proliferative phase

1								
2	0.3569							
3	0.3591	0.0000						
4	0.0261	0.2833	0.5745					
5	-0.0874	-0.0167	-0.6424	-0.0921				
6	-0.2350	0.3833	-0.0261	0.2833	0.2008			
7	0.3569	-0.4167	0.1132	-0.6000	-0.1172	-0.3500		
8	0.5396	-0.1167	0.2959	0.2500	-0.1590	<u>-0.6833</u>	0.1167	
	1	2	3	4	5	6	7	8

Early luteal phase

1								
2	0.3426							
3	0.0207	-0.2263						
4	0.2392	0.2242	0.5883					
5	-0.3567	-0.2006	0.1232	0.4863				
6	0.0970	0.0545	-0.1875	0.1879	0.5593			
7	-0.4073	-0.5515	-0.1358	0.0909	0.5654	0.2727		
8	-0.2909	0.0303	0.3426	0.1273	0.1033	-0.0182	0.2000	
	1	2	3	4	5	6	7	8

Late luteal phase

1								
2	-0.3620							
3	-0.1976	-0.0919						
4	-0.3006	-0.0909	<u>0.6833</u>					
5	-0.0838	0.1158	0.3881	<u>0.7833</u>				
6	-0.1902	-0.1152	0.1550	0.5152	0.2648			
7	0.1043	0.1879	-0.1550	-0.2242	-0.0772	0.0788		
8	0.0184	-0.3212	0.3216	<u>0.6606</u>	0.4909	<u>0.8545</u>	0.2727	
	1	2	3	4	5	6	7	8

Menstrual phase

1								
2	0.4092							
3	-0.1784	0.0838						
4	-0.4364	-0.1905	-0.4192					
5	0.0273	0.1190	-0.1078	-0.6190				
6	-0.6001	-0.0714	-0.3473	0.4524	0.1190			
7	-0.6819	-0.2857	0.1796	0.3810	-0.3333	0.1190		
8	-0.0546	0.2857	-0.0599	-0.5000	<u>0.6905</u>	0.0714	0.2381	
	1	2	3	4	5	6	7	8

b. Trimegestone

0.05 mg dose group

1								
2	0.2254							
3	-0.1824	-0.2608						
4	0.1953	0.1149	<u>0.5083</u>					
5	0.3091	-0.1246	-0.1649	0.0891				
6	0.4073	0.0278	0.1015	0.3897	0.1597			
7	-0.0754	<u>-0.4953</u>	0.0543	-0.2081	0.2446	-0.2095		
8	-0.0132	-0.1376	<u>0.5549</u>	<u>0.5667</u>	-0.2896	<u>0.4565</u>	-0.0278	
	1	2	3	4	5	6	7	8

0.10 mg dose group

1								
2	-0.0906							
3	-0.0642	0.2312						
4	<u>0.6019</u>	-0.3207	<u>-0.4508</u>					
5	0.0973	-0.3095	0.2163	0.2070				
6	0.3740	-0.1958	0.0559	0.3962	<u>0.7079</u>			
7	0.0591	-0.3058	-0.0243	-0.2268	0.0196	-0.1455		
8	0.1873	-0.0792	0.2317	-0.0629	0.0674	0.1261	0.3606	
	1	2	3	4	5	6	7	8

0.25 mg dose group

1								
2	-0.2195							
3	-0.2076	0.1226						
4	0.0056	-0.2830	0.2580					
5	-0.1512	-0.0359	0.0902	-0.2247				
6	0.1149	0.2699	0.1777	0.0134	-0.0376			
7	0.0194	-0.0284	0.2270	-0.1569	0.2377	-0.3582		
8	-0.1013	0.0840	0.1769	0.1949	0.2425	<u>0.4450</u>	-0.2583	
	1	2	3	4	5	6	7	8

0.50 mg dose group

1								
2	0.0924							
3	-0.0617	-0.3292						
4	0.1704	0.1514	0.3564					
5	0.0174	-0.3804	0.1485	-0.0310				
6	0.1362	-0.1574	0.3243	0.1204	0.2089			
7	0.0350	-0.1658	0.3158	-0.1009	0.3575	0.2973		
8	-0.2880	-0.1376	0.3988	0.2565	0.1854	0.0454	0.3070	
	1	2	3	4	5	6	7	8

Table 10.3a. Correlation between the calculated day of onset of the progestogen associated bleeding (PAB) and the apoptotic markers in the 4 doses of trimegestone. 1 = Apoptotic bodies, 2 = Bax + Stromal cells, 3 = Bcl-2 in the glandular epithelium, 4 = Bcl-2+ Stromal cells, 5 = Caspase-3 in the glandular epithelium, 6 = Caspase-3+ Stromal cells, 7= ki-67 in the glandular epithelium, 8= ki-67+ Stromal cells, 9 = Calculated day of onset of Progesterone Associated Bleeding (PAB). Underlined values = $P < 0.05$

1									
2	-0.0403								
3	-0.1521	-0.0322							
4	0.2094	-0.0561	0.2089						
5	0.0246	-0.2283	0.0653	0.0215					
6	0.2737	-0.0136	0.1494	0.1913	0.3067				
7	-0.0163	-0.2220	0.1495	-0.1643	0.1772	-0.0744			
8	-0.1274	0.0145	0.2827	0.2194	0.1182	0.3293	0.0602		
9	<u>-0.2882</u>	-0.1199	0.0177	-0.1240	0.0955	-0.0075	-0.0440	0.0500	*
	1	2	3	4	5	6	7	8	9

Table 10.3b. Correlation between the calculated day of onset of the progestogen associated bleeding (PAB) and the apoptotic markers in the 4 doses of trimegestone. Analysis of diaries of women with regular cycle length ($SD \leq 1$ day)

1									
2	0.0255								
3	-0.2620	-0.0649							
4	0.1589	0.0757	0.0940						
5	0.0268	-0.1515	0.0029	0.0229					
6	0.2193	0.0788	0.1075	0.0718	0.1761				
7	0.0865	-0.1109	0.2478	0.0132	0.0678	-0.0287			
8	-0.2879	-0.0053	0.1861	0.0110	-0.0321	0.4057	0.0363		
9	-0.2152	<u>-0.3461</u>	0.0434	<u>-0.3219</u>	0.1767	-0.1092	0.0829	0.0191	*
	1	2	3	4	5	6	7	8	9

Table 10.4: Standardised weights assigned to individual apoptotic markers along the first 4 linear discriminant functions.

Apoptotic markers	Linear Discriminant Functions			
	1	2	3	4
Apoptotic bodies (gland)	-0.12	-0.48	0.06	0.17
Bcl-2 (gland)	-0.14	-0.56	0.64	0.16
Bcl-2 (stroma)	0.07	0.31	-0.20	0.90
Bax (stroma)	-0.35	-0.02	-0.54	-0.03
Caspase-3 (gland)	-0.20	-0.00	-0.11	-0.04
Caspase-3 (stroma)	-0.05	-0.68	-0.49	-0.13
Ki-67 (gland)	-0.92	0.23	-0.23	0.01
Ki-67 (stroma)	0.32	0.17	0.16	-0.22

Chapter Eleven
The Matrix Metalloproteinases

Introduction

The connective tissue is composed of extracellular matrix (ECM), blood vessels, lymphatics and cellular components such as fibroblast and macrophages. ECM is important for cell morphology, and influences cell proliferation, differentiation and apoptosis. The degradation of the ECM is believed to be under the influence of the metalloproteinases (MMP) for a variety of features; being secreted into the ECM and function under physiological conditions; they are highly regulated and members of this family of proteins are the only enzymes capable of denaturing collagens.

There are 16 recognised types of MMPs classified under 3 subfamilies; the collagenases (MMP-1, MMP-8, MMP-2 & MMP-13), the gelatinases A and B (MMP-2 and MMP-9), and stromelysin-1 and -2 (MMP-3 and MMP-10) and matrilysin (MMP-7)²³⁴. The MMPs degrade the ECM and basement membrane in a zinc dependent fashion²³⁵. They are secreted as latent zymogens, which then become activated¹²⁷. MMP-1 (collagenase) degrade collagen I-III, VII and X, MMP-2 (Gelatinase-A) degrades gelatins, collagens IV, V, VII and XI, MMP-3 (stromelysin), degrades proteoglycan, fibronectin and laminin, while MMP-9 (gelatinase B) degrades gelatin and collagen IV and V¹²⁷. MMP-3 can activate other MMPs, such as pro-MMP-1 and pro-MMP-7, urokinase plasminogen activator and TNF- α ^{128 236}. Natural inhibitors of MMPs are either, tissue type such as TIMP 1-4 or plasma inhibitors such as α -

macroglobulins ²³⁵. TIMP-1 & 2 bind in a ratio of 1: 1 to the active site of all MMPs.

In general, the MMPs are synthesised by the connective tissue cells in the stroma, such as fibroblast, endothelial cells of newly formed vessels, macrophages, and neutrophils ¹²⁸. MMP-7 however, is secreted by cells of epithelial origin ²³⁷. MMP-1 is produced by macrophages, fibroblast and non-macrophage cells ^{238 239 240}.

Matrix metalloproteinases (MMP), are structurally related enzymes recognised as being of critical importance for the tissue breakdown at menstruation ^{127 234}. In the proliferative phase MMP-1, 3 & 9 are expressed at low levels and only focally ¹²⁸. The expression of MMPs declines in the early luteal phase, and reappears again in the late luteal phase. Most MMPs are expressed in the menstrual phase where tissue breakdown occurs. MMP-1 expression is restricted to the stromal cells mainly in the menstrual phase where it is predominant at the periphery of the shed tissue fragments ²³⁸. The expression of MMP-1 and TIMP-1 expression increase in the peri-menstrual phase, while MMP-2, TIMP-1 and -2 are present throughout the phases of the natural cycle ^{128 241}. The ratio of the activated MMPs to their inhibitors play an important role in regulating MMPs activity ¹²⁸. MMP-9 is expressed mainly in PMN, monocyte-macrophages and eosinophils¹²⁸.

In endometrial primary stromal and glandular cell cultures, a physiological concentration of progesterone reduces the protein expression of MMP-1, -2, -3, and -9, and this effect is antagonised by RU486^{242 128 126}. Oestradiol added alone inhibited the secretion of MMP-1, but the inhibition was greater when oestradiol and progesterone were administered in combination. Progesterone withdrawal from stromal cell culture increases the production of the MMPs^{243 244 245 246}. Administration of Interleukin-1 α and TNF- α for 48 hours can stimulate the endometrial stromal cells in-vitro to secrete MMP-1, -3, & -9, but not MMP-2²⁴⁷.

Progesterone is probably the principle player in the suppression of MMPs, however, the mechanism involved is not clearly understood, especially as the level of the MMPs in the natural cycle starts to decline before the level of progesterone starts to rise. Progesterone may exert its action through the activation of PAI-1²⁴⁸, TGF- β ²⁴⁹, or the stimulation of TIMP-1²⁴².

Aims

- a. To study the endometrial expression of MMP-1, -3, and -9 in response to trimegestone-based HRT in 2 doses, compared to the natural cycle.
- b. To assess the effect of trimegestone compared to progesterone on the production of MMP-1, -3 & -9 mRNA in primary endometrial stromal cell culture model.

c. To study the potential antagonising effect of mifepristone (RU486) on the expression of MMP-1, -3 & -9 in primary stromal cell cultures.

a. Immunohistochemistry

Women and methods

I elected to examine the expression of MMP-1, -3 and -9 in the endometrial samples of women who received the highest (0.5 mg, n=19) and the lowest (0.05 mg, n= 21) doses of trimegestone (page 90). Their demographic characteristics are described on page 126. The endometrial biopsies were obtained using the vabra curette on day 24 of the last treatment cycle.

The control

The characteristics of the control population (n = 32) are described on page 53. The histological diagnoses, were proliferative (n= 8), early luteal (n= 8), late luteal (n= 11) and menstrual (n= 8).

Trimegestone-treated endometrium

The technique of immunohistochemical staining for MMP-1, 3 & 9 is described on page. 58.

Assessment of the endometrial sections

The pattern of distribution of the positively stained cells was described first. To assess the number of positively stained stromal cells per field, ten randomly selected fields ¹⁶⁸ per slide were captured (x1000) under

oil immersion (page 63). All evaluated fields were restricted to the functionalis layer. Images were captured using Axioplan microscope (Carl Zeiss, Herts UK), and a colour video camera (Sony CCD/RGB). The cells counted were evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, UK). The data presented are per unit area and the corresponding areas of the images captured with x1000 were 0.0046 mm² using a measurement graticule.

b. Stromal cell culture

Endometrial stromal cells were obtained from uteri in the luteal phase of the natural cycle as described in the method section page 66. The positivity of vimentin staining of these primary cell in culture (page 67) was >99% in all cases (Figure 11.1a).

In order to assess the responsiveness of cultured stromal cells to the addition of trimegestone, and to progesterone, I assessed the production of prolactin (PRL) under the effect of progesterone²⁵⁰ using ELISA technique (Genzyme, CA, USA). Stromal cells showed an increase in PRL secretion under the effect of E₂ and P₄, and at levels higher than control. However, this was not a consistent finding, even when Relaxin (RLX) was added to the cell culture for 7 days²⁵¹.

Endometrial stromal cells in culture under the effect of MPA alone differentiated slowly and secreted PRL at about 9 days following the start of steroid treatment, while the addition of porcine RLX to MPA was associated with rapid differentiation and consequently earlier (day 7-8) and higher production of PRL²⁵¹. My data on the production of

PRL by stromal cells were variable and inconsistent. This may be due to the fact that decidualisation occurs after 9-10 days of stromal cells in culture ^{244 251}, or due to the different method I used in assessment i.e. ELISA, while others used Radioimmuno-assay ²⁴⁵ or immunocytochemistry ²⁴⁴.

Given that the design of my experiments involved different time frames to that in the published literature and that the purity of the stromal cell population in culture was the important factor to establish, I have restricted the test of stromal cell population to the vimentin expression by these cells.

In optimising the model of stromal cell cultures, the learning curve involved 10 series of experiments, and was followed by 12 series of experiments, where I optimised the methods of mRNA extraction and its purity, RT-PCR and the resolving of PCR products on agarose gel.

Primary Stromal Cell Cultures: Design of the experiments (Figure 3. 3a & 3b)

- a. Experiment series 1: E₂ (10⁻⁶M) was added for 4 days followed by trimegestone (10⁻⁶M) for 6 days, n = 6.
- b. Experiment series 2: E₂ was added for 4 days followed by either trimegestone (T) or progesterone(P₄) or 6 days, as I tried to compare the effect of equimolar concentrations of trimegestone and progesterone

(10^{-6} M) in the same primary cell cultures, using the same controls (n=6).

c. Experiments series 3a & 3b: This series was designed to demonstrate the blocking of the effects of T or P_4 by the addition of RU486 (10^{-6} M) for 4 days, with parallel cultures treated with T, P_4 , or RU486 being maintained, n = 12.

All experiments were performed in triplicate wells,

The culture medium was collected at the end of each experiment, centrifuged to remove cellular debris and stored at -80°C for subsequent analysis with ELISA (page 73). Total cellular mRNA was extracted from the stromal cells at the end of each experiment and the level of gene expression for the MMP-1, MMP-3 and GAPDH were assessed by RT-PCR. The products of the PCRs were run on 3% agarose gel, and the resulting bands were analysed as described on page 72.

Statistics

a. *Immunohistochemistry*: The data did not fulfil the assumptions necessary for using the analysis of variance and t-test, therefore the non-parametric Kruskal-Wallis and Mann-Whitney tests were used.

b. *Stromal cell culture*: The data concerning the gene expression in primary stromal cell cultures were analysed using mixed model of the log of the ratio of MMP-1:GAPDH and MMP-3: GAPDH densitometric values, incorporating subjects and treatments. Wald test was used to compare treatment effect.

Results

a. Immunohistochemistry

The stroma showed perinuclear staining, while the glands showed cytoplasmic expression of the MMPs. The cell count of endometrial stroma positive for MMP-3 in different phases of the natural cycle and in the trimegestone treated group was much lower compared to MMP-1⁺ and MMP-9⁺ cell count (Table 11.1).

a.1. *Natural cycle*

Stromal cells positive to MMP-1⁺, -3⁺ & -9⁺ were scattered in the endometrium in different phases of the natural cycle (Figure 11.1 b, c & d), with higher expression of MMP-1 and -3 in the menstrual phase, although this increase lacked statistical significance. There was a statistically significant difference in MMP-9 expression between the phases ($P = 0.034$, Table 11.1), and the highest expression was in the late luteal phase compared to the proliferative or menstrual phases ($P=0.02$, $P=0.03$ respectively). The glandular epithelial cells were 100% positive to MMP-1 and MMP-9 in all phases of the natural cycle with no apparent cycle modulation. On the other hand the glands in the different phases of the natural cycle were negative to MMP-3.

a.2. The trimegestone treated endometrium

Stromal cells positive to MMP-1, -3 and -9 were scattered in the endometrium of women treated with trimegestone-based HRT. There was no difference in the effect of the dose of trimegestone on the expression of MMP-1, -3 or -9. All the glands were positive to MMP-1 and MMP-9, while none was positive to MMP-3.

b. Stromal cell culture

Experiment series 1 (Figure 11.2a)

Trimegestone (T) suppressed MMP-1 and MMP-3 more than E_2 but, was statistically significant only in its suppression of MMP-3 ($P = 0.004$, Table 11.2a & b). T combined with E_2 led to a higher suppression of MMP-1 & MMP-3 in comparison to the control ($P = 0.000$, Figure 11.3a & b). $E_2 + T$ suppressed MMP-1 & MMP-3 to a higher degree compared to when each steroid was added alone ($P = 0.000$).

Experiment series 2 (Figure 11. 2b)

E_2 suppressed MMP-1 and MMP-3 mRNA compared to the control (Figure 11.3 c & d), but the suppression was statistically significant for MMP-1 ($P = 0.004$). Either T or P_4 alone, or in combination with oestradiol led to suppression of MMP-1 and MMP-3 mRNA compared to the control ($P = 0.0000$, Table 11.3 a & b). T alone or in combination with E_2 led to a higher suppression of MMP-1 compared to P_4 alone or $E_2 + P_4$ ($P = 0.048$, $P = 0.000$, respectively). Combined $E_2 + T$ suppressed MMP-3 mRNA more than $E_2 + P$ ($P = 0.011$), while there was no

difference in the effect of T compared to P on MMP-3 mRNA. $E_2 + P_4$ and $E_2 + T$ suppressed MMP-1 and MMP-3 mRNA significantly in comparison to when either T or P_4 was added alone ($P = 0.000$).

Experiment series 3a (Figure 11. 2 c)

Progesterone alone or in combination with E_2 suppressed MMP-1 and MMP-3 mRNA compared to the control ($P = 0.0000$, $P = 0.000$, respectively, Figure 11.3 e & f), and the suppression was higher on combined treatment of $E_2 + P_4$. This mRNA suppression of MMP-1 and MMP-3 was significantly reversed on the addition of RU486 ($P = 0.000$) which led to an increased mRNA expression of both genes. However, the increased expression of MMP-1 and -3 messages upon the administration of RU486 was still lower than the control ($P = 0.000$, Table 11.4 a & b).

Experiment series 3b (Figure 11.2 d)

Trimegestone alone or in combination with E_2 suppressed MMP-1 and MMP-3 mRNA compared to the control ($P = 0.001$, $P = 0.000$, respectively, Figure 11.3 g & h), and the suppression was higher on combined treatment of $E_2 + T$. Suppression of MMP-1 and MMP-3 by $E_2 + T$ was reversed upon the addition of RU486 to the culture ($P = 0.000$). However, the increased expression of MMP-1 and -3 mRNA upon the administration of RU486 was still lower than the control ($P = 0.009$ & $P = 0.001$, Table 11.5 a&b).

Comparison with 17 α -Oestradiol

Progesterone in combination with 17 β -oestradiol led to the suppression of MMP-1 and MMP-3 messages compared to the control (P= 0.005, P= 0.007 respectively), while there was no difference in the effect of P₄ combined with 17 α -oestradiol in comparison to the control.

Measurement of MMP-1 and MMP-3 proteins by ELISA

MMP-1 and MMP-3 proteins in culture supernatants from experiments series 3a (n= 2) and 3b (n= 6) were measured in duplicates (Data not shown). Data from set of triplicate wells were averaged and plotted against the respective treatment. All control samples contained elevated MMP-1 and -3 protein levels with an overall pattern broadly similar to the change in the message.

Summary of the study

There was higher endometrial stromal expression of MMP-1 & -3 proteins in the menstrual phase of the natural cycle, but did not reach statistical significance. MMP-9 expression was significantly higher in the late luteal phase. Endometrial glandular epithelial cells showed no cycle modulation for MMP-1 or -9.

There was no dose-response effect of trimegestone on the protein expression of MMP-1, MMP-3 or MMP-9 in the endometrial stroma of postmenopausal women treated with trimegestone-based HRT.

In primary stromal cell culture, the mRNA expression of MMP-1 and -3 genes was suppressed in cells treated with P₄ or T, which was more profound when either steroid was combined with E₂.

When the effect of T was compared to P₄ in experiment series 2, the suppression of MMP-1 mRNA was greater when either T or E₂ + T was added compared to P₄ or E₂ + P₄.

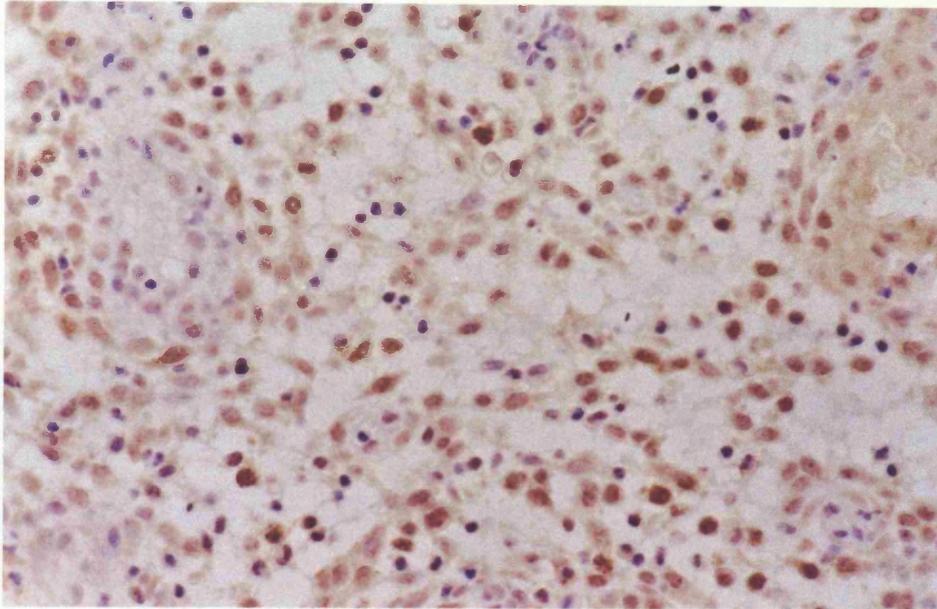
RU486 reversed the suppressive effect of P₄ and T in combination with E₂ and led to an increase in MMP-1 and MMP-3 mRNA but not completely as the levels of the respective mRNA was still lower compared to the control.

The supernatant from the experiment series 3a & 3b contained MMP-1 and MMP-3 proteins in a pattern broadly similar to the changes observed in the respective mRNA. The levels of MMP-3 protein however, was 3 orders of magnitude higher than those of MMP-1.

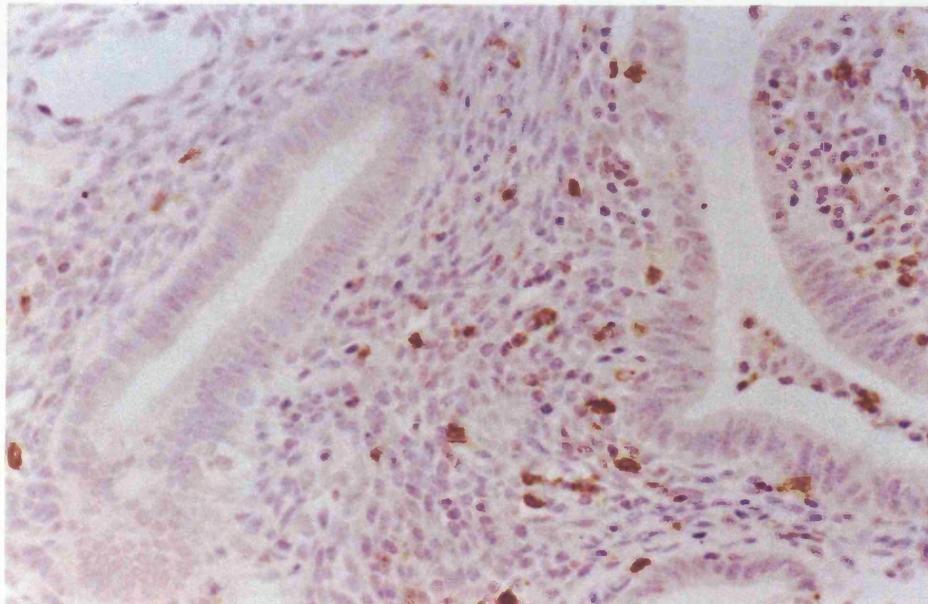


11.1a

Figure 11. 1a. Immunohistochemical staining of endometrial stromal cells with vimentin, 1cm = 100 μ m.

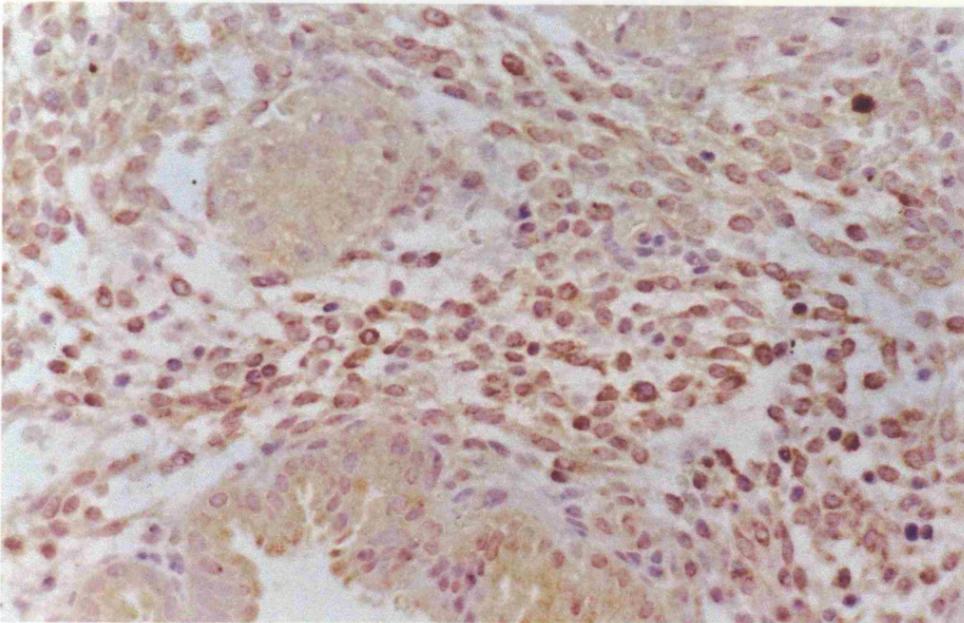


11.1b



11.1c

Figure 11.1b & 1c: Immunohistochemical staining of endometrial sections in the late luteal phase showing MMP-1 staining of the stroma, original magnification x 200, 1cm = 100 μ m (1b). Immunohistochemical staining showing MMP-3 in the stroma, original magnification x 200, 1cm = 100 μ m (1c).



11.1d

Figure 11.1d: Immunohistochemical staining of endometrial sections in the late luteal phase showing cytoplasmic staining with MMP-9 of the stroma, original magnification x 200, 1cm = 100 μ m.

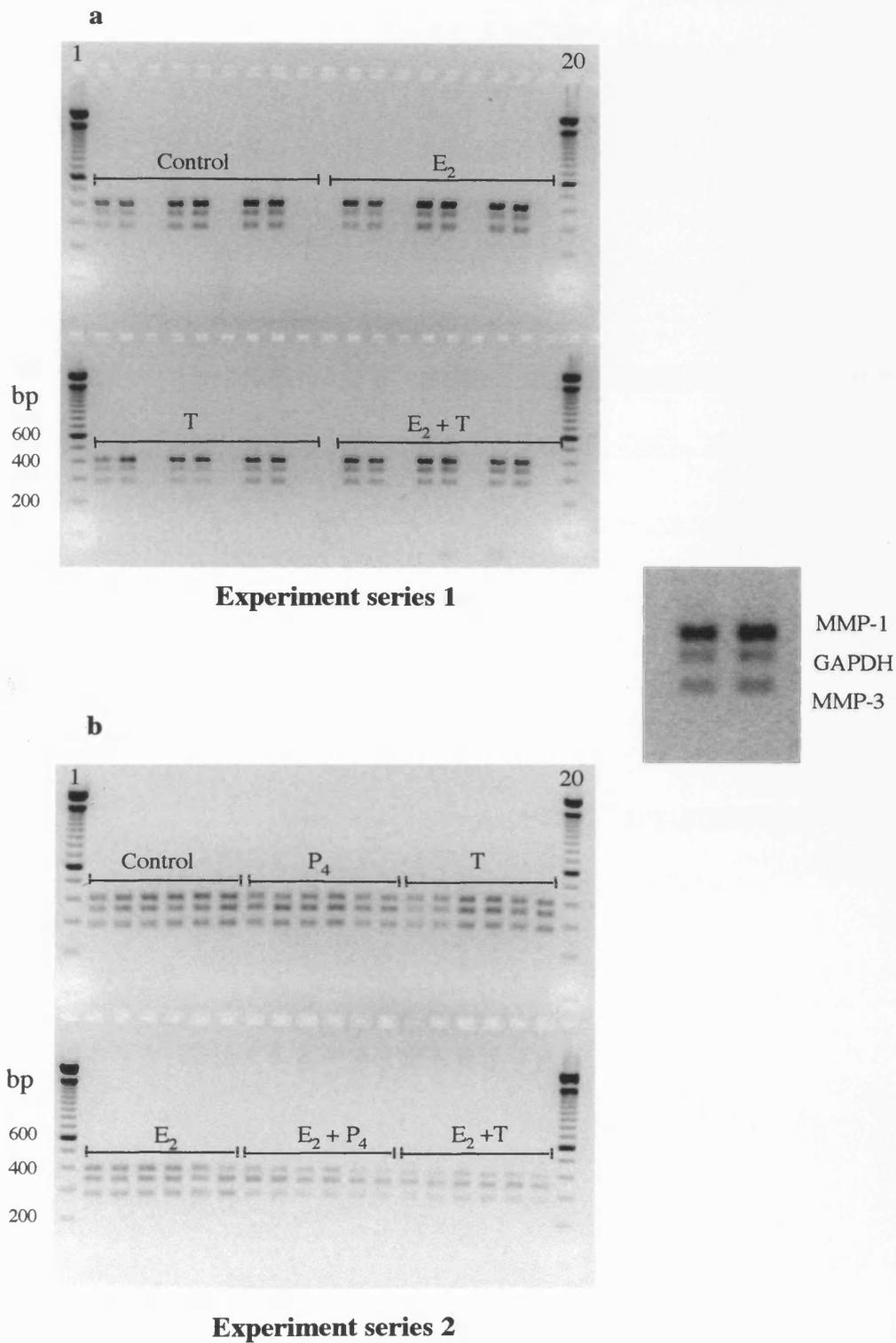


Figure 11.2a & b: The resolving cDNA bands of MMP-1, MMP-3 and GAPDH in experiment series 1 (a), and 2 (b). Lanes 1 & 20 = 100bp ladder. E₂ = Oestradiol, P₄ = Progesterone, T = Trimegestone.

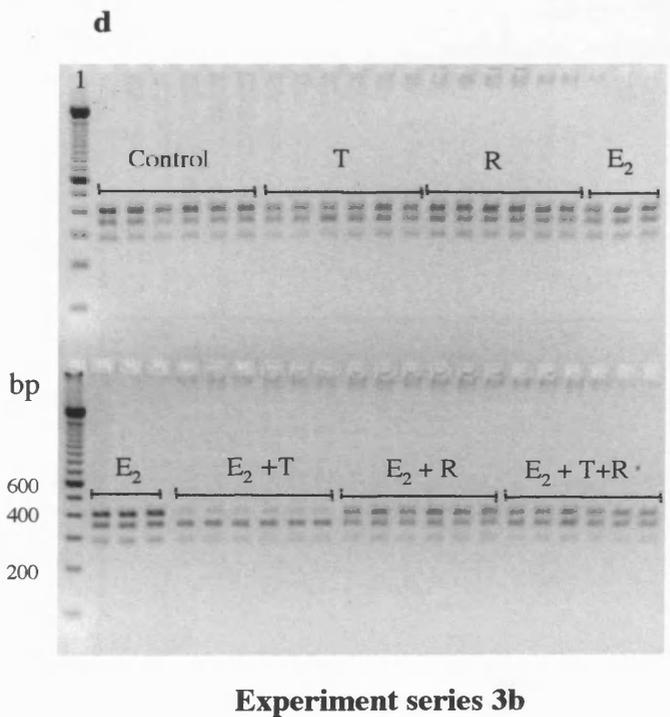
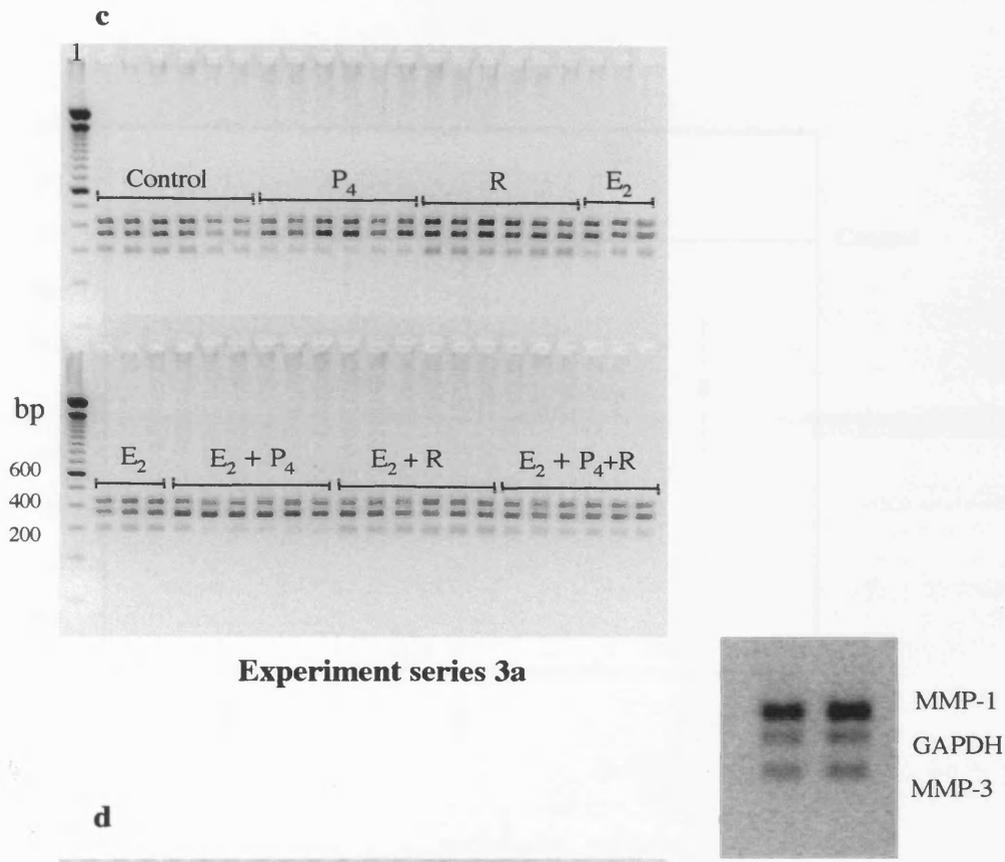


Figure 11.2c & d: The resolving cDNA bands of MMP-1, MMP-3 and GAPDH in experiment series 3a (c), and 3b (d). Lane 1 = 100bp ladder. E₂ = Oestradiol, P₄ = Progesterone, T = Trimegestone, R= RU486.

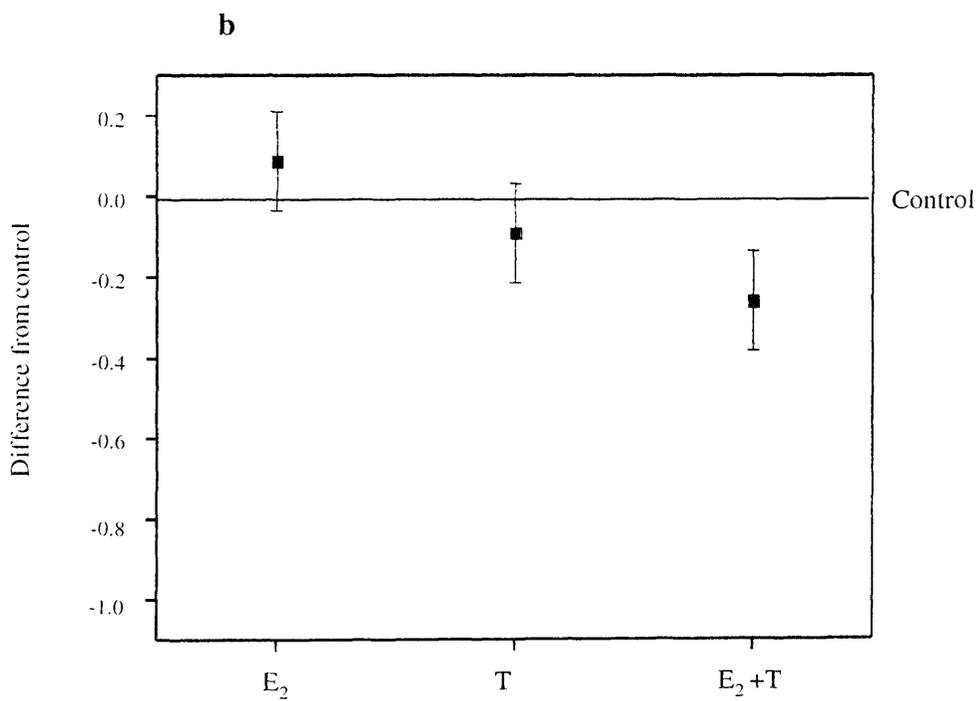
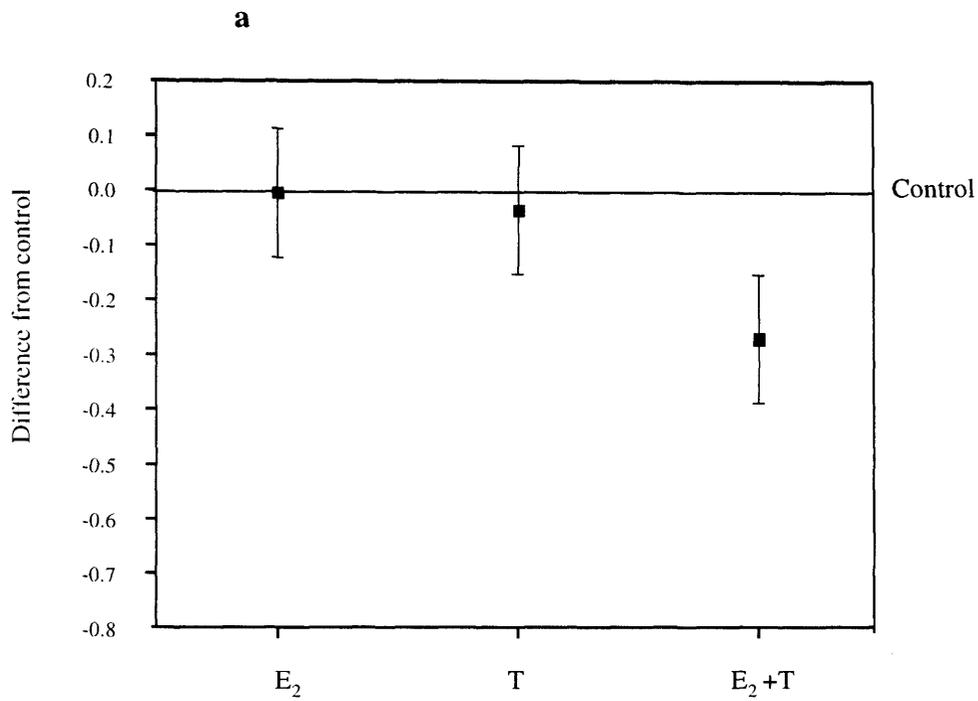


Figure 11.3a & b: The difference from the control (mean \pm SEM) of the log ratio of MMP-1/GAPDH (a) and MMP-3/GAPDH (b) in experiment series 1. Statistically significant comparisons in mRNA expression were noted between different treatments and the control, as well as among individual treatments (page 256). E₂ = Oestradiol, T= Trimegestone

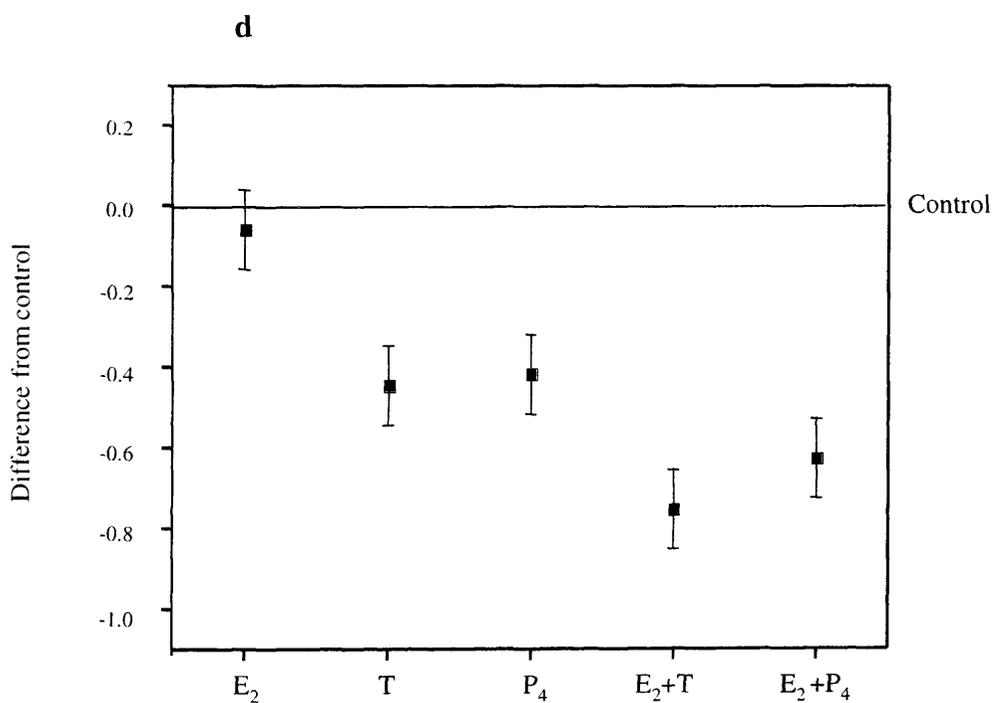
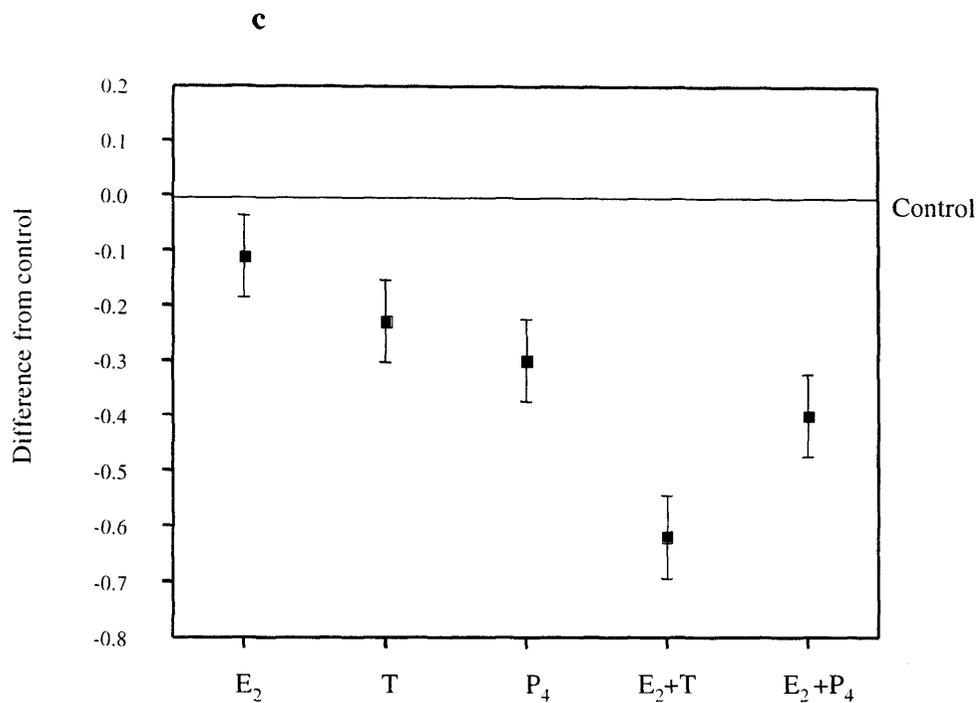


Figure 11.3c & d: The difference from the control (mean \pm SEM) of the log ratio of MMP-1/GAPDH (c) and MMP-3/GAPDH (d) in experiment series 2. Statistically significant comparisons in mRNA expression were noted between different treatments and the control, as well as among individual treatments (page 256). E₂ = Oestradiol, T = Trimegestone

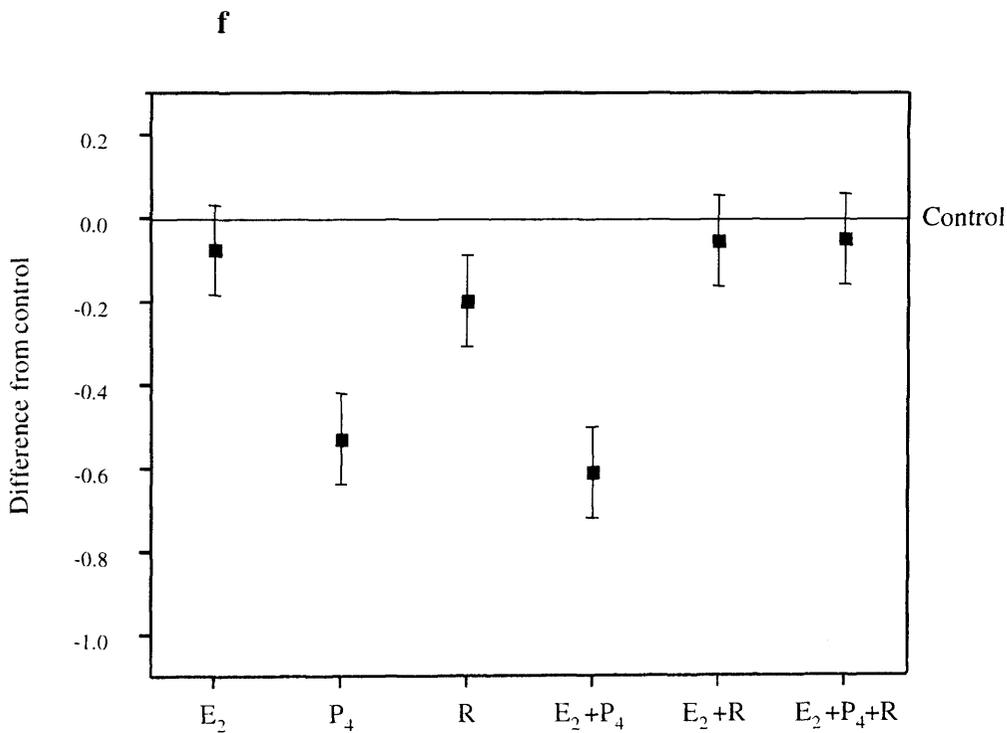
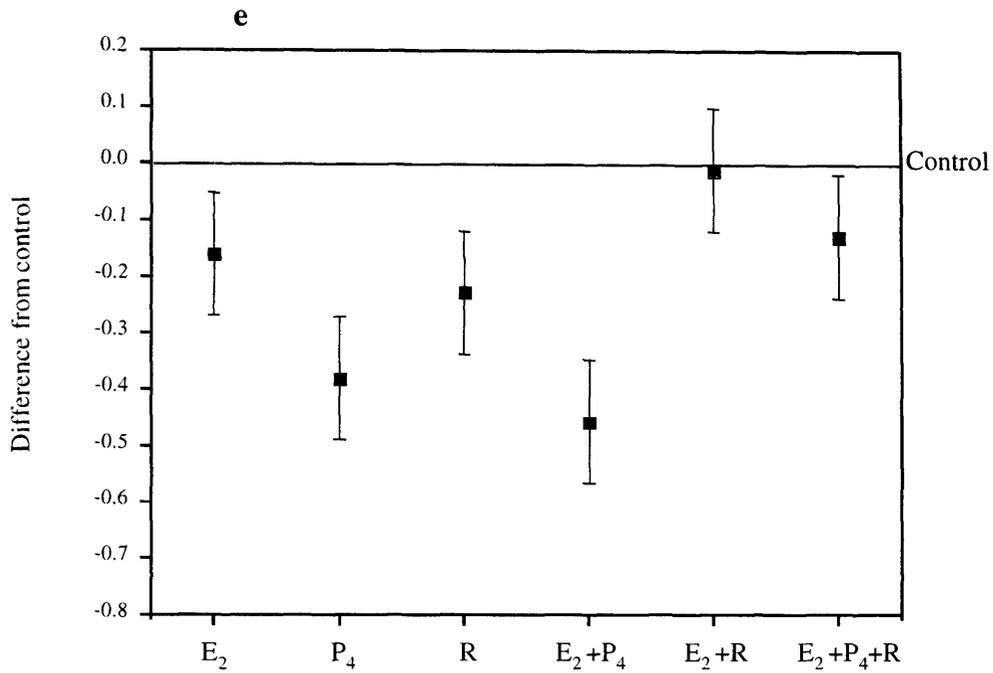


Figure 11.3e & f: The difference from the control (mean \pm SEM) of the log ratio of MMP-1/GAPDH (e) and MMP-3/GAPDH (f) in experiment series 3a. Statistically significant comparisons in mRNA expression were noted between different treatments and the control, as well as among individual treatments (page 257). E₂ = Oestradiol, P₄ = Progesterone, R = RU486

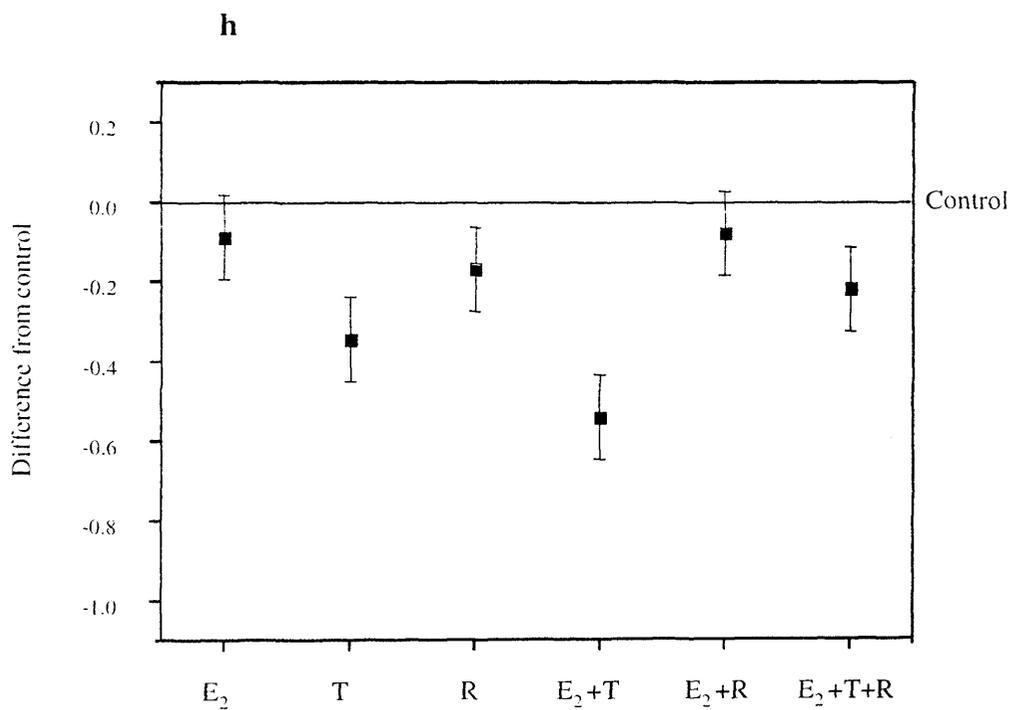
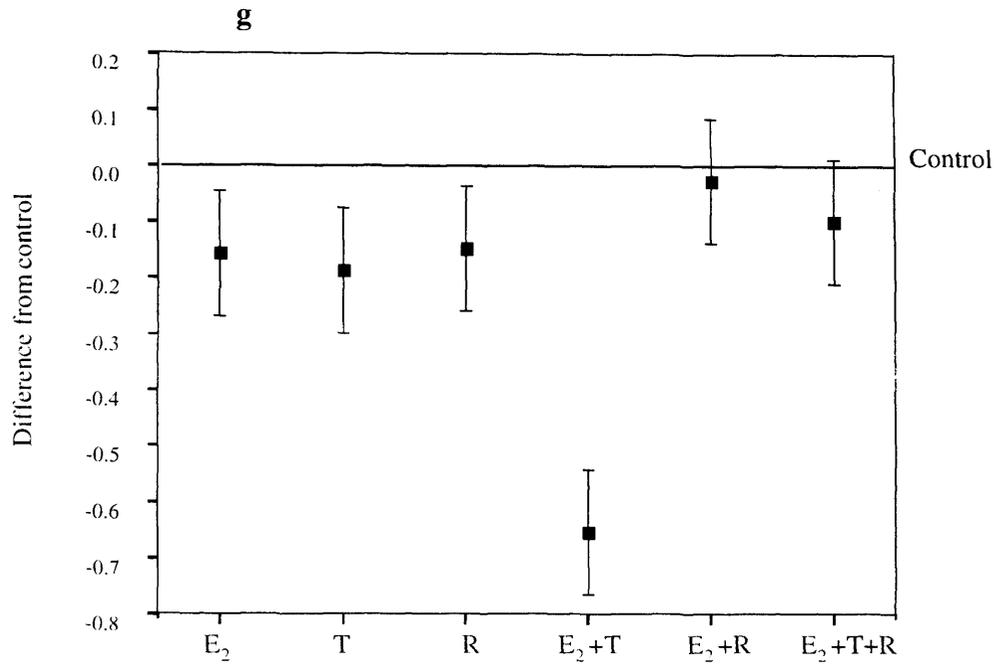


Figure 11.3g & h: The difference from the control (mean \pm SEM) of the of the log ratio of MMP-1/GAPDH (g) and MMP-3/GAPDH (h) in experiment series 3b. Statistically significant comparisons in mRNA expression were noted between different treatments and the control, as well as among individual treatments (page 257). E₂ = Oestradiol, T= Trimegestone, R= RU486

Table 11.1. Mean and standard deviation (SD) of number of the stromal cells positive to MMP-1, MMP-3 and MMP-9 in the endometrium of the natural cycle and the endometrium treated with 2 doses of sequential trimegestone. Data are per mm² endometrial tissue.

Mean (SD)	Endometrium of the natural cycle				Trimegestone	
	P	EL	LL	M	0.05 mg	0.5 mg
	n = 8	8	11	8	21	19
MMP-1	6582 (2544)	5165 (2163)	6969 (2633)	7594 (5227)	9028 (2613)	8306 (3320)
MMP-3	374 (494)	573 (794)	451 (328)	698 (482)	423 (734)	719 (1032)
MMP-9	3305 (1947)	5351 (1780)	6425 (2892)	3447 (1740)	2128 (2127)	2409 (2275)

P = proliferative, EL = early luteal, LL = late luteal, M = menstrual

Table 11.2a. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-1/GAPDH in experiment series 1.

	Mean of log	SE	Geometric mean
Control	0.36	0.04	1.43
Oestradiol (E ₂)	0.35	0.06	1.43
Trimegestone (T)	0.32	0.13	1.38
E ₂ +T	0.09	0.10	1.09

Table 11.2b. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-3/GAPDH in experiment series 1.

	Mean of log	SE	Geometric mean
Control	-0.03	0.05	0.97
Oestradiol (E ₂)	0.06	0.04	1.06
Trimegestone (T)	-0.12	0.16	0.89
E ₂ +T	-0.29	0.28	0.75

Table 11.3a. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-1/GAPDH in experiment series 2.

	Mean of log	SE	Geometric mean
Control	0.23	0.04	1.26
Oestradiol (E ₂)	0.12	0.04	1.13
Progesterone (P ₄)	-0.07	0.03	0.93
Trimegestone (T)	0.00	0.05	1.00
E ₂ + P ₄	-0.17	0.07	0.84
E ₂ +T	-0.39	0.08	0.68

Table 11.3b. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-3/GAPDH in experiment series 2.

	Mean of log	SE	Geometric mean
Control	0.08	0.05	1.08
Oestradiol (E ₂)	0.02	0.03	1.02
Progesterone (P ₄)	-0.34	0.07	0.71
Trimegestone (T)	-0.37	0.18	0.69
E ₂ + P ₄	-0.55	0.15	0.58
E ₂ +T	-0.67	0.14	0.51

Table 11.4a. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-1/GAPDH in experiment series 3a.

	Mean of log	SE	Geometric mean
Control	0.05	0.06	1.05
Oestradiol (E ₂)	-0.11	0.08	0.89
Progesterone (P ₄)	-0.33	0.12	0.72
RU486	-0.18	0.13	0.84
E ₂ + P ₄	-0.41	0.07	0.67
E ₂ + RU486	0.04	0.04	1.04
E ₂ + P ₄ + RU486	-0.08	0.05	0.92

Table 11.4b. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-3/GAPDH in experiment series 3a.

	Mean of log	SE	Geometric mean
Control	-0.15	0.03	0.86
Oestradiol (E ₂)	-0.22	-0.06	0.80
Progesterone (P ₄)	-0.68	0.09	0.51
RU486	-0.35	0.04	0.71
E ₂ + P ₄	-0.76	0.07	0.47
E ₂ + RU486	-0.20	0.10	0.82
E ₂ + P ₄ + RU486	-0.20	0.05	0.82

Table 11.5a. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-1/GAPDH in experiment series 3b.

	Mean of log	SE	Geometric mean
Control	0.28	0.04	1.33
Oestradiol (E ₂)	0.13	0.07	1.14
Trimegestone (T)	0.10	0.05	1.10
RU486	0.14	0.06	1.15
E ₂ + T	-0.37	0.21	0.69
E ₂ + RU486	0.26	0.03	1.29
E ₂ +T+ RU486	0.19	0.07	1.20

Table 11.5b. Mean and standard error (SE) of the log of the ratios and geometric mean ratio of MMP-3/GAPDH in experiment series 3b.

	Mean of log	SE	Geometric mean
Control	0.00	0.05	1.00
Oestradiol (E ₂)	-0.10	0.04	0.90
Trimegestone (T)	-0.35	0.12	0.71
RU486	-0.17	0.04	0.84
E ₂ + T	-0.55	0.21	0.58
E ₂ + RU486	-0.08	0.03	0.92
E ₂ +T+ RU486	-0.22	0.08	0.80

Chapter Twelve
General discussion

My research started on the basis of clinical observations of the unscheduled bleeding in postmenopausal women who received sequential combined HRT regimens. I examined clinical parameters that might characterise such women. The data collected on a double blind, dose-ranging study of a new progestogen, trimegestone, provided me with the opportunity to ask the relevant clinical and scientific questions in a well defined group of postmenopausal women randomly allocated to receive one of 4 doses of the progestogen with a single dose of oestrogen.

Effect of the dose of trimegestone

The dose of trimegestone was the major determinant of the pattern of uterine bleeding. Higher doses were associated with progressively “later” mean day of onset of bleeding, with reduced variability between cycles compared to women who had received the lower doses of trimegestone, whose mean day of onset of bleeding was early during the progestogen phase and varied considerably from cycle to cycle. Women on the higher doses had shorter and lighter PAB. This interesting association of a later onset of bleeding episodes and a shorter duration suggests fuller differentiation of the endometrium prior to shedding with higher trimegestone doses. “Early” onset bleeding and the association with a longer duration of blood loss may reflect regional differences in endometrial maturation and regular shedding of the endometrium with a lower dose of the progestogen.

The within cycle variability of the day of onset of bleeding, concerns many HRT users and many women will be keen to find out when the next bleed will be. I calculated the chance of transition from a given day of onset of bleeding in a given cycle into an earlier or a later day of onset in a subsequent cycle. Women who bled before the end of the progestogen phase, regardless of the dose of trimegestone, had a higher incidence of early bleeding in the next cycle; conversely "later" onset bleeding in one treatment cycle was more likely to be associated with late onset of bleeding in the next cycle. For example, a day of onset on or earlier than day 28 in the current treatment cycle was associated with a 74% chance for a similar day of onset of PAB occurring in the next cycle. The finding of a later onset of bleeding in the treatment cycle being associated with a more predictable pattern of bleeding is similar to a previous report ⁶¹. This suggests that this method of predicting the day of onset of bleeding can form the basis of a clinical advice and the patient herself may be able to anticipate the day of onset of bleeding in the next cycle when using a particular preparation of HRT.

The higher incidence of IMB, which appeared to be of shorter duration as the dose of trimegestone increased, could be due to the progressive delay in the onset of bleeding with higher doses, which included extension of the bleeding episodes outside the PAB window. In the second 6 months, when the dose of trimegestone was fixed at 0.25 mg, women who were on the highest dose (0.5 mg) had a lower incidence of IMB, but experienced earlier mean day of onset of the PAB at 29.5 day

instead of 30.9 day. This shift in the pattern of bleeding supports the assumption that the high incidence of IMB in the 0.5 mg trimegestone dose group in the first 6 months may be due to the extension of bleeding outside the PAB window, thus considered as IMB. However, the frequency of IMB was too low to permit further interpretation of this increase.

Since the favourable changes in lipoprotein profile in the 0.25mg trimegestone dose group were documented ²⁵², the sponsoring pharmaceutical company decided to choose this dose in an extension phase of the study of further 6 months. This is despite the fact that the bleeding episodes were more tightly regulated in 0.5 mg trimegestone dose group. This change of doses of trimegestone in the extension study has given me the opportunity to study the clinical aspects of endometrial bleeding upon changing the dose of the progestogen, in addition to the effect of duration of use in those who continued on the same dose of trimegestone.

The effect of the dose of trimegestone remained dominant when women continued the study for a further 6 cycles but received a fixed dose of trimegestone (0.25 mg). Women who were on the lower doses of 0.05 mg and 0.1 mg and then started the higher dose of 0.25 mg had a better pattern of bleeding. Women who were on the 0.5mg dose and then changed to 0.25mg regressed to have less satisfactory bleeding pattern.

It has been accepted that with the increased duration of treatment, the incidence of abnormal bleeding episodes decreases^{253 65}, although most of the times, it is difficult to prove in a controlled study due to withdrawal of patients who experience such bleeding episodes. In this study, however, there was no improvement in the incidence of abnormal bleeding patterns in women who continued on the 0.25 mg dose for a further 6 months. Moreover, these women experienced a higher incidence of IMB compared to the first 6 months and there was no higher frequency of submucous fibroids as assessed hysteroscopically, compared to other treatment groups. This phenomenon remains unexplained.

The Role of structural abnormalities involving the endometrium

Since some of the women on the higher doses of trimegestone experienced early onset, heavy and prolonged PAB, and others who received the lower doses had late onset and light menstrual discharge of short duration, other factors must be invoked in regulating menstrual shedding. This may be due to individual differences in hormone bioavailability, although serum levels of trimegestone for women in the study were in the expected range, for the 4 dose groups (Data on file, Hoechst Marion Roussel). Nevertheless, the effect of endometrial structural abnormalities, submucous fibroids and endometrial polyps, on the pattern of bleeding needed assessment.

The pathogenesis of abnormal uterine bleeding in association with submucous fibroids is not fully understood and a number of explanations have been suggested. Sampson (1912) found that there were structural and branching abnormalities in the arterial blood supply to these fibroids, although an obstruction to the uterine venous plexus could lead to generalised or focal endometrial venous ectasia and consequently more prolonged and heavier blood loss²⁵⁴. This however remains an unconfirmed possibility. Further, it is a common observation that the endometrium overlying submucous fibroids shows less advanced luteal changes compared to the adjacent endometrial tissue, and it is plausible that such features may contribute to unscheduled bleeding.

The presence of submucous fibroids was associated with prolonged and heavy PAB with a higher incidence of IMB. The effect of submucous fibroids on the duration and severity of the PAB episodes was not apparent in the lowest dose of trimegestone (0.05mg), which may be due to fewer women with submucous fibroids in this group.

Alternatively, there may be a dose threshold, below, which the dose response relationship no longer exists. It was not possible to adequately evaluate a potential interaction between an individual dose of trimegestone and the presence of submucous fibroids in view of the small number of women with submucous fibroids in each dose group. Similarly, the number of women with submucous fibroids in each dose

group was too small to allow a separate analysis of the effect of the number and grade on the pattern of bleeding.

The lack of association of unscheduled bleeding with endometrial polyps agrees with previous reports^{104 106}. Although in a case report, endometrial polyps were the only endometrial pathology found in women with levonorgestrel laden intra uterine device who experienced breakthrough bleeding. However, these data related to only 3 women²⁵⁵.

In Chapter 5, I used a different definition of the bleeding episode to that in Chapter 4, as on further analysis I felt that many women would have considered any bleeding episode following a bleeding-free interval, even of 1 day, as undesirable. The dose of trimegestone in this data set had no effect on the incidence of IMB, while the presence of submucous fibroids predicted its occurrence. Nevertheless, while the presence of submucous fibroids was associated with abnormal bleeding pattern, the effect of the dose of trimegestone was still the dominant factor.

Endometrial histomorphometry

Histological assessment of the endometrial biopsies was performed at the end of 6 treatment cycles, and despite the dose dependent modulation of the timing and severity of the bleeding episodes, about 96% of these biopsies showed secretory changes, with no statistically

significant difference between the 4 dose groups. This agrees with Sturdee et al. (1994) who reported on the histological findings of endometrial samples obtained from 413 postmenopausal women using different sequential HRT preparations for the previous 3 months, and found no correlation between the timing of the withdrawal bleeding and endometrial histology ¹⁸².

Trimegestone was administered for 14 days, and the duration of progestogen administration for 10-14 days is widely acceptable in clinical practice as protective of the endometrium ^{175 176}. Recently, Weiderpass et al. (1999) ⁴¹ reported that the sequential combination of oestrogen and progestogen administration for fewer than 16 days per 28-day treatment cycle was associated with an increase in the relative risk of endometrial hyperplasia and carcinoma. Out of 256 randomised women into the dose ranging study of sequential trimegestone-based HRT, only one biopsy showed simple hyperplasia in a woman who had been receiving 0.5 mg trimegestone. This is below the incidence seen in an untreated population of postmenopausal women ²⁵⁶ and may have occurred by chance. Inadequate endometrial biopsy for histological diagnosis was encountered in 4 cases, and in all 4 the uterine cavity was documented to be atrophic by hysteroscopy. This suggests that this type of progestogen, administered for 14 days out of 28 days cycle, confer an adequate endometrial safety profile.

The principle of dating the endometrium among histopathologists relies on assigning the stage of development to the most advanced feature of the endometrial section being examined. In order to overcome this largely subjective evaluation of the endometrium, I applied histomorphometric methods in order to explore potential effects of the dose of trimegestone on endometrial development and to compare the whole group of tissue samples to those of the natural cycle.

In the natural cycle, the mean total glandular area and the average glandular diameter were higher in the luteal phase, the height of the glandular epithelium was lower in the mid luteal phase, while there was no difference in the number of glands, which agrees with the published literature ^{257 187}.

The smaller mean total glandular area in the endometrium of trimegestone-based HRT, compared to the natural cycle agrees with the published literature on other sequential HRT regimens, however, the average glandular diameter was smaller, while there was no difference in the average glandular number, which is at variance to the findings reported using cyclical sequential oestradiol and NET-based HRT regimen ⁵¹. This may be due to the different progestin administered in this study which has no affinity to oestrogen receptors and minimal androgen receptor affinity, while NET has a potent androgen receptor affinity in addition to progesterone receptor binding. Therefore, NET

may exert a different effect on glandular tortuosity with different number of sectioned glands appearing per unit area. Moreover, NET, through its androgen receptor binding may differentially affect the endometrium through the increase of EGF receptor expression, thus enhancing the action of EGF on endometrial glandular growth and differentiation ²⁵⁸. Such regulation of EGF expression may be directly relevant, since EGF is also involved in endometrial angiogenesis. Lastly, the possibility exists of the potential effect of conversion of NET to EE ⁴⁵, thereby augmenting the oestrogenic stimulation of the endometrium.

The highest dose of trimegestone (0.5mg/day) was associated with lower glandular secretions. This may be similar to the picture of “aborted secretion” described by Dallenbach-Hellweg ⁵³, and may indirectly explain the more favourable pattern of bleeding in this group in comparison to women on the lower doses ²⁵⁹.

I assessed the glandular area and diameter in 2 dimensional images, although the assessment of the endometrial glands in a 3-dimensional model may theoretically provide more information on endometrial development ²⁶⁰. However, such method extrapolates the glandular dimensions, for example, from sections obtained at different levels of the tissue block, and as such adds further hypothetical assumptions which may reduce the power of the analysis.

There was no significant difference in the stromal cellularity between the five phases of the natural cycle, which is at variance to the reports by Johannisson et al. (1987)²⁵⁷, and Dockery et al. (1990)²⁶¹. The latter group found an increase in the number of stromal nuclei per unit volume of stromal cells in LH + 2 to +6, but decreased in day LH + 6 to +8. However, Noyes et al. (1950), encountered a similar apparent paradox created by intercellular oedema, where stromal cells appeared less in number due to the small, dense nuclei⁵². Stromal cellularity included the stromal cells and the infiltrating leukocytes. Stromal leukocytes increased in the late luteal phase by about 150 %, nevertheless, even if I allow for this increase, it will only have a minimal effect on the total stromal cell count. Indeed, in a separate analysis, the addition of the leukocyte common antigen positive (CD45⁺) cell count for the whole series of specimens failed to add a significant effect on linear discriminant functions (data not shown). To my knowledge there is no similar data on stromal cell count in the published literature to which my findings can be compared. The endometrium of women treated with sequential trimegestone-based HRT showed no effect of the dose of trimegestone on stromal cellularity.

Casanas-Roux et al. (1996)¹⁸⁸, studied the vascularisation of the endometrium under the effect of cyclical sequential HRT using natural progesterone-containing vaginal gel. They reported slight increase in the capillary : stroma relative surface area in the HRT cycle, though was

not significant. These findings were not confirmed in my study, as there was statistically significantly smaller mean total vascular space area and diameter in the HRT treated endometrium compared to the late luteal and menstrual phases. This may be a special effect of trimegestone-based HRT, which, while having no androgen receptor binding activity, is not identical to the natural progesterone either.

In this study the only difference between the endometrium of women who bled on the day of the biopsy compared to those who had not, was more glandular invagination (telescoping) in the latter group; which may be due to the fact that these glands have been shed during the bleeding. Many pathologists, nevertheless, argue that glandular telescoping is merely the result of mechanical disruption of the glands during fixation and cutting ⁵⁵.

I realise that the trimegestone -treated endometrial samples obtained in this study represent a “snap shot” of histological changes at a fixed time point of the progestogen administered, day 10 (day 24 of the treatment cycle), but my intention was to compare the extent of the progestogen effect in influencing endometrial differentiation. As such the weighted values of histological parameters calculated in this study appear to be dose independent, and therefore they may represent a specific effect of trimegestone, number of days being administered, or both. Further the effect of individual variation in oestrogen bioavailability to the

endometrium may account at least in part for the histological variations, but this possibility remains speculative.

The direct comparisons of individual histological parameters failed to illustrate a determinant factor which characterises the trimegestone-treated endometrium. Therefore, in the presence of a mixed picture of endometrial development under HRT regimens and the wide inter-specimen variability, I evaluated the relative weighting of these histological parameters by adopting linear discriminant analysis (Chapter 7, page 146)

Linear discriminant analysis of the 13 histological variables examined, helped to distinguish between the endometrium of the treated groups and the 5 phases of the natural cycle. The endometrium of the natural cycle is distinguished from the trimegestone treated endometrium in the first rotated linear discriminant function by larger mean total glandular and vascular areas, and lower percentage of glands with telescoping (Figure 7.3a). The second rotated linear discriminant function distinguishes between the proliferative and the early luteal phases (Figure 7.3b). The proliferative phase is characterised by larger mean total vascular space area but smaller average vascular diameter. Conversely the early luteal phase is associated with large mean total glandular area, and small average glandular diameter, in addition to higher percentage of glands with vacuolations. As the mean number of the glands do not differ in the early luteal from the other phases of the

natural cycle, then the larger mean total glandular area with the smaller average glandular diameter is probably due to the increase in glandular tortuosity, which changes glandular shape.

Apart from scantier glandular secretions in the higher dose groups, there was no difference in the other histological parameters between the 4 dose groups, which does not explain the 4 different bleeding patterns of the 4 dose groups of trimegestone. This agrees with Li et al. (1992) who reported that endometrial development might be mediated by mechanisms other than the dose of progesterone ⁷².

Endometrial leukocytes

Endometrial leukocytes have been implicated in the process of endometrial bleeding and shedding. I assessed the leukocytic infiltration in the endometrial biopsies of these women and compared the results to those of the natural cycle. The distribution and pattern of expression of CD45⁺, CD56⁺ and CD3⁺ cells and the increased count of these cells in the late luteal and menstrual phases of the natural cycle, as well as the pattern of expression of Ki-67⁺ cells are in agreement with the published literature ^{194 195 204 262 92}. There is a lack of agreement in the literature, however, as to the precise number of endometrial leukocyte and their subsets in any particular phase of endometrial development. A number of reasons may explain these differences: the tendency of endometrial leukocytes to aggregate around glands which necessitates the examination of more fields per specimen, and when in

aggregate, it may pose a practical difficulty in counting the leukocytes individually. Other factors include different glandular density, or varying degree of stromal oedema, in addition to variation in antigen retrieval methods and the source and specificity's of the antibodies used.

In counting the positively stained leukocytes per unit area I did not make allowance for the size of the glands or for stromal oedema, where the number of stromal cells per unit area is reduced. However the fact remains that the function of these lymphomyeloid cells is not dependent on the actual number of stromal cells, since there is a net increase in the number of endometrial leukocytes in the late luteal phase of the natural cycle ¹⁴⁷.

Song et al (1995) ¹⁹¹ reported that a dose-response relationship existed between the administered progestogen (NET and MPA) and the expression of the CD45⁺ and CD3⁺ cells in endometrial stroma which were significantly higher than the control. In the trimegestone-treated women, there was no dose-response effect of trimegestone on the expression of CD45⁺, CD56⁺, CD3⁺ leukocytes or Ki-67⁺ cells.

In order to identify differences between the HRT-treated endometrium and that of the natural cycle, I have utilised a linear discriminant analysis model. In this model, the number of CD45⁺, CD56⁺ and CD3⁺ cells distinguished the trimegestone-treated endometria from the late

luteal and menstrual phases of the natural cycle, while the number of Ki-67⁺ and the doubly labelled Ki-67⁺/CD45⁺ cells distinguished these endometria from the proliferative and early luteal phases of the natural cycle. None of these markers, however, distinguished between the endometrial samples treated with any of the 4 doses of trimegestone. The reason why trimegestone is having a different effect on leukocyte infiltration is not clear, but may well be related to a different pattern of induction of progesterone modulated chemotactic factors by stromal cells such as RANTES protein ²¹² and MCP-1 ²¹¹.

There were a higher number of endometrial leukocytes in women who were bleeding at the time of the biopsy which agrees with the proposed function of these cells in endometrial disintegration and shedding. Similar results were reported by Clarke et al (1996) in their study on women treated with levonorgestrel implant (Norplant) who experienced irregular bleeding and showed an increase in CD3⁺ and CD68⁺ cells in the endometrium obtained from those women ²⁶². Critchley et al (1998), reported an association between LNG IUCD and increase CD56⁺ infiltration, but similarly, there was no correlation between the bleeding pattern and the endometrial changes induced by LNG ²⁶³.

There is no agreement in the literature regarding the origin of the increase of these leukocytes. In my study, the increase in the endometrial leukocyte population is probably the result of migration

rather than local proliferation, since only 1:7 of the endometrial leukocytes were positive for the proliferation marker Ki-67. This is at variance to other studies^{264 265 266 150} who attributed the increase in the leukocyte infiltration to an increase of in-situ proliferation and this may well be related to the type of progestogen used in their studies.

I believe that the generally reduced total cell count for CD45⁺ and Ki-67⁺ cells in sections stained by the double labelling technique compared to those singly labelled for each antigen, is due to the added steps of antigen retrieval and washings necessary for the double labelling technique, since the pattern of endometrial expression of these antigens is similar in both techniques.

Endometrial vascularity

Given the fact that the dose of trimegestone did not influence the cell count for CD45⁺, CD56⁺, CD3⁺, Ki-67⁺ and CD45⁺/Ki-67⁺ cells in the endometrium, whether the woman had started bleeding by the day of the biopsy or not; the endometrial infiltration with these cells did not explain the different patterns of bleeding in these postmenopausal women. Vasculogenesis has been shown to change during different phases of the natural cycle and therefore the possibility of a direct steroid modulation led me to examine this compartment of the endometrium using monoclonal antibody raised against CD34 antigen in an immunohistochemical approach. The following account summarises the evidence for the potential influence of sex steroids.

New vessel formation involves degradation of the basement membrane by the action of collagenase and plasminogen activators secreted by the endothelial cells. Endothelial cells then, migrate through these openings formed in the basement membrane as a loose sprout. A lumen is formed by curvature of the endothelial cells, followed by division of the endothelial cells, and then canalization as the sprouts join each other ²⁶⁷. The major stimulus of angiogenesis is hypoxia ²⁶⁸ and increased metabolic demands ²⁶⁹, but the exact mechanisms involved are not known.

Capillaries, including those of the endometrium, are lined by a continuous single layer of endothelial cells arranged over a basement membrane. Pericytes surround some of these endothelial cells which form projections that make contact with endothelial cells. Capillaries lack a smooth muscle layer, however capillaries and small vessels (consist of 1 or 2 endothelial cells) may stain for α -smooth muscle actin (α -SMA) ^{270 271}. Ultrastructurally, endothelial cells show changes in activity and size according to the phase of the menstrual cycle ²⁷². Oestrogen and progesterone receptors have not been found in endometrial endothelium ⁹¹, which is at variance to the findings of Iruela-Arispe et al. (1999) ²⁷³. Therefore, it is plausible to postulate that steroid modulation of changes in cellular activity and morphology may be mediated indirectly through changes in extracellular matrix proteins ²⁷⁴, integrins ²⁷⁵, or through the induction of cytokines such as vascular

endothelial growth factor (VEGF), basic fibroblast growth factor, bFGF²⁷⁶, epidermal growth factor,²⁷⁷ or transforming growth factor alpha, TGF α ²⁷⁸.

VEGF exists in 5 isoforms expressed in the endometrium, but only VEGF165 and VEGF121 variants are shown to be modulated by sex steroids¹³⁰. Evidence of increased VEGF expression has been documented in polycystic ovaries²⁷⁹, and in ovarian hyperstimulation syndrome¹³¹. VEGF isoforms are present in the extracellular matrix and attached to proteoglycans on the cell surface. VEGF may act in a paracrine/ autocrine manner as small patches of immunostaining are found around some blood vessels, in the glandular epithelium and in the stromal cells²⁸⁰. VEGF acts synergistically with angiopoietin-1, to induce sprout formation²⁸¹.

Basic FGF is expressed in glandular epithelial and stromal cells, although there is no change in expression across the phases of the natural cycle^{276 282}. However, there is increased glandular and stromal expression of bFGF in simple and complex hyperplasia, where it may stimulate the synthesis of plasminogen activator by the endothelial cells^{282 283}. The involvement of extracellular matrix proteins²⁷⁴ was highlighted by the finding of cycle regulation of thrombospondin-1 in the endometrium. Thrombospondin-1, a multifunctional extracellular matrix glycoprotein, is a suppresser of angiogenesis, in-vitro and in-vivo, and is regulated by progesterone²⁸⁴.

The expression of CD34 in the endometrial microvasculature defined different vascular parameters to those assessed in the H&E sections. Endometrial vascular space area and diameter were statistically significantly smaller in the early luteal phase compared to the late luteal or menstrual phases, a different finding to that obtained from examination of the H&E sections. The use of CD34 staining technique is more accurate in identifying blood vessels compared to H&E staining, where it is sometimes difficult to identify the microvasculature, which when constricted or small may blend with the surrounding stromal cells ^{219 285}. CD34 antigen is also expressed on endothelial cells of lymphatics, however there is no indication in the literature that sex steroids modify lymphangiogenesis.

Rogers et al. (1993) ²¹⁸ reported a similar decrease in the number of vascular spaces in early luteal phase, and an increase in the menstrual phase, using anti-CD34 antibody; although their findings were not statistically significantly different from the other phases of the natural cycle. Ota et al. (1998), studied the vascularity in the endometrium of the natural cycle, and found significant increase in the total vascular surface area, number of capillaries and the mean diameter in the luteal phase compared to the proliferative phase, which is at variance to my findings ²²¹. This may be due to the less specific polyclonal antibody for von Willebrand factor used in that study.

There was no difference in the vascular parameters studied in the 4 dose groups of trimegestone, while NET-based HRT was associated with a significantly higher number of smaller vascular spaces than in the trimegestone-treated endometrium or that of the natural cycle.

There were no statistically significant differences in vascular measurements in endometrial samples obtained from women who had bled on the day of the biopsy compared to those who had not bled by then, in both HRT regimens.

Increased microvascular density was noted in endometria of women treated with levonorgestrel subdermal implants ²¹⁸, and suggested uncoupling of microvascular and extravascular compartments. On the other hand, medroxyprogesterone acetate administration was found to suppress angiogenesis in endometrial cancer transplants experiments ²⁸⁶, and suppressed fibroblast growth factor activity in cultures of endometrial cancer cells ²⁸⁷. Orally administered MPA was shown to reduce microvascular density in women with endometrial hyperplasia ²⁸⁸.

A known difference between LNG and MPA acetate is that the former poses a much higher androgenic effect than the latter. In this study, trimegestone with the characteristic of very low androgen receptor binding behaved in a manner similar to progesterone in the natural cycle, as far as the vascular morphometric parameters studied. The androgenic progestogen, NET, on the other hand, affected microvascular density in a manner analogous to subdermal LNG implants, although

LNG administration was continuous and not cyclical. This may suggest that androgen receptor activation probably play an important role in modulating angiogenesis.

Regional variation in endometrial development could be another factor for the observed difference, in endometrial vascular parameters between trimegestone and NET-treated endometria. Shaw et al. (1979) reported a lower mean number of blood vessels in the isthmus compared to the fundus, corpus, or cornua, however they doubt the statistical significance of their finding ²¹⁹. In this study, the endometrial biopsies from women treated with NET-based HRT, were obtained from the corpus region of hysterectomy specimens (area B, page 76), while in the trimegestone groups a vabra curettage was performed, which would sample tissues from different parts of the uterine cavity. Therefore, such differences would have been expected to result in a lower number of vascular spaces compared to the NET treated endometrium, and as such the issue of regional differences in endometrial development do not explain the prevalence of smaller vascular diameter in the latter group.

There is a difference in the duration of the 2 types of HRT regimens with the trimegestone group treated for 6 months, while NET-based HRT was given for 3 months. There is no information in the literature on vascular morphometry as defined by CD34, over time. However, Hickey et al. (1999) found that most of the changes in the components of the endometrial vascular basement membrane induced by Norplant

reverted back to normal after 12 weeks of treatment. Nevertheless, it should be pointed out that the essential difference was the continuous administration of levonorgestrel in the study by Hickey et al. (1999) compared to the cyclical sequential administration of the progestogens in this study²⁸⁹.

The apoptotic and anti-apoptotic markers in the endometrium

So far, I could find no demonstrable effect of the dose of sequentially administered trimegestone on the histomorphometric features, pattern of leukocytic infiltration and its subtypes population, or on vascular morphometric analyses of endometria thus treated, for 6 months. With the clinical behaviour of the endometrium during the dose ranging study and during the extension phase, so strongly dependent on the dose of trimegestone, I furthered my investigations into potential initiators of tissue disintegration; namely apoptosis regulating signals.

It is hypothesised that apoptosis is involved in the process of menstruation^{157 225}. Tabibzadeh et al. (1995) ascribed the increased prevalence of apoptosis in the late luteal and menstrual phases to the concomitant rise in TNF- α expression, which may be involved in the extrinsic pathway of caspase activation cascade.

There is a lack of agreement in the literature as to the distribution of the apoptotic bodies-containing cells in the endometrium of the

natural cycle. These cells were found only in glandular epithelium with no cycle variation ²⁹⁰, in glandular epithelium of the basalis layer during the late luteal and menstrual phases ²⁹¹, in the stroma of the functionalis during the luteal phase ²²⁵, while Toki et al. (1998), found the expression mainly in the glands of the basal layer during the late luteal phase ¹¹¹. In my study, I found no statistically significant difference in the endometrial glandular expression of the apoptotic bodies-containing cells in different phases of the natural cycle.

The number of bcl-2⁺ glandular epithelial cells was higher in the proliferative endometrium compared to the other phases of the natural cycle, which agrees with the published literature ^{292 293 294 166}, but at variance was the stromal cells' expression of bcl-2 in the late luteal phase. Bcl2 was reported as weakly expressed in the luteal phase and none was expressed in the proliferative phase ²⁹², while McLaren et al. (1997) observed no stromal immunoreactivity throughout the menstrual cycle ¹⁶⁶, and Koh et al. (1994) found minimal and infrequent expression in the proliferative and early luteal phases with increased immunoreactivity in the late luteal phase ²⁹⁴.

The prevalence of apoptotic bodies in glandular epithelium and of stromal expression of bcl-2 in this study contrast with the published literature and may be due to different definitions of samples, since the stringent criteria for sample inclusion in my study are not evident in those reports. Further, the examination of fewer fields than in this

study may be responsible for these observed differences. The different patterns of expression of bcl-2 in the glands and stroma in different phases of the natural cycle are suggestive of hormonal regulation of this protein. The expression of bcl-2 has been strongly correlated with oestrogen receptor expression in primary breast adenocarcinoma cell cultures thus furnishing a further evidence for the hormonal regulation of this protein ²⁹⁵.

The expression of bax is not hormonally modulated, as there is no difference in the glandular or stromal expression between the phases of the natural cycle, which agrees with the report on its expression in the endometrium ¹⁶⁶ and in breast tissue ²⁹⁶.

Krajewski et al. (1994) found a reciprocal pattern of expression of bcl-2 and bax in the lymph nodes and the colon ¹⁶⁴. The relative expression of bcl-2 and bax may determine the propensity of cells for apoptosis ²⁹⁷ ²³⁰ ²³¹ ¹⁶¹. Given that bax is not modulated during different phases of the natural cycle, unlike bcl-2, it is plausible to postulate that bax may exert its function through an altered rate of hetero-dimerisation with bcl-2 or through an alternative pathway.

The expression of bcl-2 and bax is cytoplasmic in the glands and stroma, while caspase-3 is expressed in the cytoplasm of the glandular epithelium but in the nuclei of stromal cells. Similar findings were reported by Krajewska et al. (1997), where cytosolic staining was noted in the secretory cells lining the lumina of the prostatic glands, but

occasionally the nuclei of these cells were caspase-3⁺ ¹⁶⁷. Further, this group described caspase-3 immunoreactivity in the cytosol of colonic epithelial cells, but some of these cells, especially those producing excess amount of mucous, had the staining concentrated in the nuclei. In addition, they found that the caspase-3⁺ cells were negative to the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining, and therefore concluded that the nuclei of these cells were not apoptotic. TUNEL technique is not the preferred method of assessment of apoptosis, not only because nuclear fragmentation is a late event in the process of apoptosis, but there is the possibility of overestimation of apoptosis depending on the method of detection used, such as microwaving ²⁹⁸. If TUNEL is used to assess apoptosis, then another marker should be used in addition. My findings were similar, when I used the microwave oven for the TUNEL technique, and unacceptably high incidence of TUNEL⁺ cells were encountered (data not shown).

Being an effector, caspase-3 is responsible for protein degradation in the cytosol and cell organelles. There is an increased stromal expression in the menstrual phase, which coincides with endometrial disintegration and shedding. There is an increased expression of caspase-3 in the glandular epithelial cells of the proliferative phase, where ki-67 expression is at its highest, but tissue destruction does not occur in this phase of the natural cycle. This may be due to the inhibition of caspase-3 activation by the mitochondrial membrane stabilising effect of bcl-2,

which is highly expressed in this phase. Both proteins, however, are increased in the stroma of the menstrual phase, although the number of caspase-3⁺ cells is much higher in the stroma. It is not clear, however why bcl-2 suppression of apoptosis is as effective as during the proliferative phase. An increase in the number of cells ready to undergo apoptosis due to higher caspase-3 expression per se, may be an oversimplification. Nevertheless, it may suggest a role for caspase-3 in the process of menstruation since its activation may occur following intracytoplasmic calcium fluxes ²⁹⁹ in the ischaemia- reperfusion model observed by Markee (1940) ⁹⁸.

The highly statistically significant correlation between the expression of caspase-3 in the glands and stromal expression of bcl-2, ki-67 and caspase-3 of the natural cycle do not lend themselves to a direct biochemical relationship in the life-death signals balance, and may represent cellular features which develop as a result of downstream effects of genes activation regulated by the prevailing steroid milieu. Similarly, the positive correlation between some of the markers in the 0.05 and 0.1 mg doses of trimegestone are interesting observation but difficult to explain.

To assess whether it is the duration of progestogen administration that determines the pattern of expression of these markers, I stratified the data on trimegestone-treated endometria according to the expected day of onset of the bleeding using the formula previously reported ²⁵⁹. I

found that the later the “expected” day of onset of bleeding, the lower was the expression of the apoptotic bodies, bcl-2 and bax. This furnishes a further evidence that the balance of expression of markers of life-death signals are more relevant to the stage of endometrial development rather than the duration of progestogen use.

Further, in the linear discriminant analysis model, the first linear discriminant function dominated by ki-67⁺ glandular epithelial cells distinguished the trimegestone-treated endometria, irrespective of the dose, from the proliferative and early luteal phases of the natural cycle. The second linear discriminant function dominated by the glandular apoptotic bodies, bcl-2⁺ glandular epithelial cells and caspase-3⁺ stromal cells distinguished the proliferative from the early luteal phase.

The possibilities are that either apoptosis is not the initiating mechanism for endometrial shedding; or that the pro-apoptotic signals may overpower the anti-apoptotic factors resulting in cell death of varied extent. Since the extent of mitochondrial permeabilisation appears to be the rate-limiting step of apoptotic and necrotic cell death depending on the extent of uncoupling, or inhibition of the respiratory chain³⁰⁰; a threshold for the number of cells involved in cell death processes may be reached that favours the induction of MMPs by necrotic cells setting off the chain of events that lead to endometrial disintegration. It will be very interesting to examine the endometrium

of women who miss episodes of withdrawal bleeding between HRT cycles to assess the prevalence of apoptosis.

The process of endometrial disintegration probably starts first in the stroma with degradation of the extracellular matrix, while the disintegration of the glands is the end results of loosening of the stroma. The prevalence of apoptosis among CID-9 mammary epithelial cells grown on tissue culture plastics was suppressed when these cells were cultured on basement membrane ECM³⁰¹. Such suppression was reversed when anti- β_1 integrin was added, suggesting that the promotion of cell survival depended on interaction with components of ECM. In this model neither fibronectin, nor type I collagen helped to suppress apoptosis. Further, when stromelysin-1 expression was induced in these cells cultured on filters where these cells deposit their own ECM, it resulted in apoptosis, which was inhibited upon the addition of metalloproteinase inhibitor (GM6001). Moreover, degradation of extracellular matrix induces the expression and activation of interleukin Converting Enzyme (ICE, caspase-1), which leads to apoptosis in-vitro and in-vivo probably through the activation of apical caspases such as caspase-8.

The administration of high doses of a progestogen in the late luteal phase can delay menstruation, which may involve the suppression of MMPs activation. Nevertheless, in women using HRT, bleeding may occur before the end of the progestogen phase, which is suggestive of involvement of processes upstream of MMP activation. Alternatively,

given the relatively short clearance time of progestogens, their bioavailability to the endometrium may be compromised, especially when low doses are used, by multitude of variables related to compliance or dietary factors. This may create a state similar to progestogen withdrawal, thus destabilising the endometrium. Therefore, I assessed the expression of MMP-1, 3 & 9 in the trimegestone-treated endometrium and compared that to the natural cycle.

The metalloproteinases

Immunohistochemistry

In the endometrium of the natural cycle, MMP-1 and -3 were expressed in all the phases but were highest in the menstrual phase, however, at variance to other studies the difference was not statistically significant^{128 302 238}. This may be due to the nature of immunohistochemistry with different steps of antigen retrieval and antibodies used were obtained from different suppliers to those studies.

MMP-9 on the other hand, was expressed in the endometrial stroma in all phases of the natural cycle with higher expression in the late luteal phase before a decrease in the menstrual phase, which agrees with the findings by Skinner et al. (1999)³⁰³. Jaziorska et al. (1996) on the other hand, reported stromal expression of MMP-9 only 2 days before the onset of and during menstruation, and in association with endometrial polymorphs³⁰². The latter group used dual immunolocalisation, and

found that MMP-9 was expressed mainly by the leukocytes in the menstrual phase.

The number of MMP-3⁺ stromal cells was much lower in all phases of the natural cycle and in the trimegestone-treated endometria compared to the count of MMP-1⁺ or MMP-9⁺ stromal cells, which agrees with previous reports, where MMP-3 was expressed in less than 2% of the stromal area ³⁰⁴. It is not clear whether the lower count is due to a limited role of this MMP in the process of endometrial degradation, or is it because MMP-3 acts indirectly through activation of MMP-7, which in turn activates MMP-9 ³⁰⁴.

There was no dose-response effect of trimegestone on the protein expression of MMP-1, MMP-3 or MMP-9 in the endometrial stroma of postmenopausal women treated with trimegestone-based HRT. In addition, there was no correlation between the pattern of bleeding in the trimegestone treated women and the expression of MMP-1, -3 or -9 which agrees with the literature ²⁰⁷.

In a comparative study of endometria treated with LNG implants (Norplant), DMPA for 12 months, or control samples obtained from the late luteal and the menstrual phases, a significantly higher expression of MMP-1 was noted in the Norplant-treated endometrium compared to those exposed to DMPA or the control. MMP-1⁺ and MMP-3⁺ stromal cells were present in small foci of cells distributed

throughout the endometrium of progestogens-treated women, and were not confined to the area of tissue breakdown during the perimenstrual phase as in the control group ³⁰⁴. Skinner et al. (1999), found no difference in the endometrial expression of MMP-9 in the glandular epithelium between the different phases of the natural cycle, and the expression was higher than that in the stroma ³⁰³. However, endometria exposed to LNG intrauterine device for 12 months showed a reversed immunostaining characteristics, where the stroma showed a higher immunoreactivity of MMP-9 than the atrophic glands. In both studies, LNG had a different effect on the distribution of MMP-1, -3 in the endometrial stroma ³⁰⁴ and MMP-9 in the glandular epithelium ³⁰³, compared to the natural cycle. While in my study, there was no difference in the immunoreactivity of MMP-1, -3 and -9 between the trimegestone-treated endometrium and that of the natural cycle. This may be due to the minimal androgen receptor affinity of trimegestone compared to the androgenic characteristic of LNG.

Primary stromal cell culture

At this point I found it necessary to ask the question as to whether the similarities detected in the immunohistochemical staining of these MMPs in the endometrial samples reflected a “genuine” feature of a progesterone-like action of trimegestone. I elected to study primary stromal cell cultures since these cells were shown to produce MMP-1, -3 and -9 and the histological changes in the endometrium of the late

luteal and menstrual phases of the natural cycle usually show more advanced changes of stromal tissue breakdown compared to the glandular compartment. Moreover, stromal cells may be more sensitive to the effect of progesterone and other progestogens, as well as to their withdrawal.

For homogeneity and to reduce the variability between specimens, all tissues for stromal cell culture were chosen from the luteal phase. Although, Rawdanowicz et al. (1994) has shown that there was no correlation in MMP production by stromal cells in culture between the phases of the natural cycle ²⁴⁷.

I adopted the model of initial propagation of freshly isolated stromal cells for one passage and then seeding the cells into 6-well plates. The cells identity was confirmed by positive vimentin staining which was consistently confirmed in more than 99% of the cells. The cells, after the first passage, were subjected to a fixed phase of oestrogen (E_2) priming followed by the addition of progesterone (P_4) or trimegestone (T), either alone or in combination with oestradiol. Further sets of experiments were conducted using RU486 as a method of P_4 or T, "withdrawal" at the receptor level. In the experiment series 1, I studied the effect of adding trimegestone to the stromal cells in culture, while in experiment series 2 the effect of T was compared to the natural P_4 in each sample. Experiment series 3, was designed to study the effect of reversal of P_4 , or trimegestone, action with RU486.

In these primary stromal cell cultures, mRNA expression of MMP-1 and -3 genes was suppressed in cells treated with P₄ or T, but this suppression was more profound when either steroid was combined with E₂. In experiment series 2, the suppression of MMP-1 mRNA was greater when either T, or E₂ + T, was compared to P₄ or E₂ + P₄. RU486 reversed the suppressive effects of both progesterone and trimegestone and led to an increase in MMP-1 and MMP-3 mRNA but not completely as the levels of the respective mRNA was still lower compared to the control.

In all the experiments I have conducted, the most interesting findings were that the levels of MMP-1, and MMP-3 mRNA and their respective proteins were higher in the control wells compared to the sex steroid treated wells. This has not been reported in the literature, as either the control was not commented on, or the addition of oestradiol alone was used as a control. This may explain, at least in part, why long term stromal cell cultures can be maintained only under high concentrations of the progestogen MPA, for example ³⁰⁵.

The inhibition of production of MMP-1 and MMP-3 mRNA under the effect of P₄ and the progestogen, T, agrees with the published literature on other progestogens ^{246 244 245}. Hampton et al. (1999) studied the effect of natural progesterone and synthetic progestogens (MPA, NET, LNG, ORG2058) in combination with oestradiol in endometrial stromal cell cultures for 15 days, or combined treatment for 10 days followed by 5

days of progestogen withdrawal ²⁴⁶. All progestins, natural and synthetic, inhibited MMP-1 and MMP-3, but increased TIMP-1 production compared to cell cultures exposed only to oestradiol.

In experiment series 2, each sample of endometrial stromal cells was subjected to the effect of T or P₄ in combination with oestradiol, T led to a higher suppression of MMP-1 mRNA compared to progesterone. This may well be due to the much higher affinity of trimegestone to the progesterone receptor and more protracted ligand-receptor binding. In a similar study, LNG and NET, characterised by a high androgen receptor binding affinity, induced a similar suppression of the MMPs to that induced by progesterone ²⁴⁶. The involvement of the androgen receptor, therefore, seems to be an unlikely explanation.

Trimegestone and progesterone were administered for a short period of 6 days and this time course was adequate to show the inhibitory effects of progesterone and trimegestone on MMP-1 and MMP-3 mRNA (initial optimisation of the experiments, data not shown). This suppressive effect of P₄ has been shown to be maintained for more than 15 days ²⁴⁶. In earlier experiments of similar design to those of experiment series 3a and 3b, the cells started to peel off the well plate on day 5 following P₄ withdrawal or the addition of RU486, and therefore I discontinued these experiments. Whether this was due to the release of the MMPs was not clear, but other studies were able to reverse the effect of MPA by the addition of RU486 for 5 days ²⁴⁵.

Salamonsen et al. (1997), suggested that withdrawal of progesterone alter the ratio of MMPs to TIMPs, favouring the breakdown of endometrial tissue. This assertion was based on the observation that in-vitro stromal cell cultures exposed to oestradiol and progesterone treatment for 6 days, followed by withdrawal of progesterone for 4 days was associated with an increase in MMP-1, -2, -3 and -9 with no effect on TIMP-1, -2 or -3 ²⁴⁴.

To reverse the progestogenic effect on stromal cells in culture, either the progestin is withheld for 4-5 days ^{244 245 246}, or RU486 is administered for 4 days ²⁴⁵. RU486 competes with P₄ at the level of the receptor binding site in the ligand binding domain, leading to a different transformation of the receptor ³⁰⁶. It is interesting that in stromal cell culture, withdrawal of different progestins on day 10 resulted in increased MMP-1 production 5 days later, except for the withdrawal of LNG where MMP-1 production was not restored. On the other hand, progesterone withdrawal, resulted in different responses of increase or no change ²⁴⁶.

In the experiment series 3a and 3b, supplementation of RU486 to the stromal cells in culture resulted in an increase MMP-1 and MMP-3 mRNA which agrees with the published literature ²⁴⁵, although this increase was still lower compared to the control. This may be due to an incomplete antagonising effect of RU486, which I used, in equimolar

concentration (10^{-6} M) to progesterone and trimegestone. Schatz et al. (1997) for example, used RU486 at 10-folds higher concentration to reverse the effect of MPA, and concluded that reversal with RU486 is 10 fold more effective than by just withdrawing MPA from stromal cell culture ²⁴⁵. Nevertheless, this group did not compare the withdrawal of MPA from cultures to the addition of equimolar concentration of RU486.

The phenol free growth medium used in these experiments was supplemented by charcoal stripped fetal calf serum, thus removing steroids and growth factors found in the serum, since my intention was to study the effect of steroids added in specified concentrations. I did not add growth factors to these stromal cell culture, although Rawdanowicz (1994) demonstrated an increase in MMP-1, -3 and -9 in stromal cell culture following the addition of TNF- α and IL-1 α ²⁴⁷. Such experiments may reflect the contributory effects of cytokines to the induction of genes including those of MMPs, and are not necessarily linked to the withdrawal of P₄ or other progestogens.

The purpose of measurement of MMP-1 and MMP-3 protein levels in the supernatants was to identify whether a similar transcription profile existed as a result of changes in the respective mRNA. This was generally the case, but it was difficult to draw a direct comparison between the two, since the number of cells was variable in these wells, while the level of mRNA for the individual MMP was evaluated

against GAPDH. In addition, a time lag could exist from the change in the level of the message and the respective protein produced. This may explain the discrepancy between the elevated messages for MMP-1 and -3 compared to their respective proteins in wells treated with $E_2 + P_4 +$ RU486 or $E_2 + T +$ RU486. In only 2 sets of experiments, either combination of steroid led to a rise in MMP-1 and -3 proteins compared to the elevated mRNA levels in all of them.

In all experiments reported in this thesis, the inhibitory effect of P_4 , or T, on MMP-1 or -3 mRNA was higher when combined with oestradiol than when administered alone, and might well be due to the upregulation of the progesterone receptor(s) by oestradiol.

The variability between samples has been experienced by other authors (Hampton 1999). This is a common feature of clinical samples, which are obtained from different women who have different genetic make-up.

For example, differences in the length of dinucleotide repeat (TATA) in the promoter region of the oestrogen receptor α^{307} result in different levels of oestrogen receptor expression, and consequently an altered pattern of expression of genes downstream, such as the progesterone receptor (s). Speculative though it may be, it is tempting to suggest that such factors may contribute to the observation of different bleeding patterns experienced by women treated with the same progestogen. Research in this field will continue to elucidate initiating factors responsible for endometrial instability, which result in abnormal or

unscheduled bleeding. Not only will such endeavours help to optimise treatments of postmenopausal women, but will have far reaching influences on the management of menstrual disorders and fertility control.

Future Research

The initiating factors of the phenomenon of uterine bleeding remains ill defined, and considering its impact on quality of life of present day women, it deserves further investigations. Since steroid receptors activation by various ligands can stimulate the transcription of myriad genes downstream, techniques such as microarray may identify other genomic loci being responsible for the intricate differences in endometrial growth and development. In collaboration with the MRC toxicology unit in Leicester, I will examine these stromal cells under different sex steroid treatments in an attempt to establish characteristic patterns of genes being activated by different ligands. When genes, activated and suppressed are identified, they can be analysed in more details and in particular their protein products can be searched for.

Given the ubiquitous nature of oestrogen receptors throughout the body, and given the fact that progesterone and other progestogens down-regulate the oestrogen receptors, it is necessary to examine the cellular changes subsequent to the concomitant administration of progestogens, in all body tissues. Not only will this help to avoid long term adverse effect as a result of long term use of HRT, but will also help to understand many pathophysiological processes as modulated by sex steroids.

Endometrial angiogenesis and its subsequent breakdown form an essential part of the bleeding phenomenon. The maturity of the

capillaries formed at the functionalis may be impaired resulting in vascular fragility and consequent endometrial bleeding. Identification of factors responsible for capillary fragility and impaired maturity may be a key factor in the understanding of endometrial bleeding.

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Appendix A : Solutions and reagents

Citrate buffer

9 ml of 0.1M citric acid
41 ml sodium citrate
450 ml of dH₂O.

PBS, Phosphate buffered sulphate.

80gm NaCl
13.7gm Na₂HP0₄.7H₂O
2gm 1.4 mM KH₂P0₄
500ml distilled water (dH₂O)
Adjust pH to 7.6
Add dH₂O up to 1000ml

TBS, Trisma Buffered Saline

71g tris
85g NaCl
%00ml water
adjust the pH to 7.6
Mgcl 4g
Water up to 1000ml.

Proteinase K (10µg/ml)

10µl of 1mg/ml solution of proteinase K in dH₂O (Boeringer Mannheim Biochemicals, UK)
990µl 0.05M TrisHCl pH 7.6

Tris (0.1M, pH 9.5)

1.2 of tris powder in 85 ml UP H₂O

0.05% Tween 20 (Sigma)

1ml of Polyoxyethylene-sorbitan Monolaurate
2l PBS

Pepsin 0.4% HCL

0.4gm/100ml Porcine pepsin 1:2500 (Sigma) /dH₂O
200µl 5M Hydrochloric acid

Mayers Hematoxylin

1gm Hematoxylin
50gm aluminium potassium sulphate
0.2gm Sodium iodate 1gm Citric acid
1litre dH₂O

Gel loading buffer (5XGLB)

20mg Bromophenol blue
8ml sterile ultrapure water (UP water) in sterile bottle.

2ml of 50XTAE
10ml glicerol
Mix and store in fridge

To use in PCR reaction dilute further:

2ml 5XGLB
4ml 50XTAE
12ml glicerol
2ml UP water

Dynabeads oligo (dT) 25

This is a suspension of 5mg beads / ml of PBS, containing
0.05% Tween-20 and 0.02% NaN₃ as preservative.

Lysis binding buffer

100mM Tris.HCL pH 8.0		5ml
500mM LiCl	1.06g	
10mM EDTA pH 8.0	1ml	
1% LiDS (SDS)		5ml
5mM dithiothreitol (DTT)	250µl	

In final volume of 50 mls DEPC water

Washing buffer with LiDS:

10mM Tris.HCL pH8.0
0.15M LiCl
1mM EDTA
0.1% LiDS

Washing buffer without LiDS:

10 mM Tris.HCL pH8.0
0.15M LiCl
1mM EDTA

TAE buffer X50 (Tris acetate EDTA)

484 tris (hydroxyl (methyl) methylamine)
1200 up water
200ml of 0.5 EDTA
114.2ml glacial acetate acid
Mix then make up to 2 litres

TBE buffer 10XTBE

0.9 Mtris-borate
0.01MEDTA
108g Tris-base
55g boric acid
20ml 0.5M EDTA

Di ethyl pyrocarbonate (DEPC) solution or water

Add DEPC (Sigma) to the solution to 0.1% (v/v). Shake vigorously or stir for 2 hours to dissolve and leave to stand overnight in a fume hood. Boil for 30 minutes. All steps are performed in fume hood.

Treat glassware etc. by soaking in 3% hydrogen Peroxide for 1 hour, and dry in a fan oven at 37°.

Paraformaldehyde 0.4%

4g in 100 ml 10xPBS

Solutions used in stromal cell culture

Collagenase, made up of mixture of;

1. 0.25 g collagenase type I (GibcoBRL, Paisley, Scotland)
2. 100 ml HBSS (GibcoBRL, Paisley, Scotland)
3. 1 ml antibiotic/antimycotic (Penicillin 10.000, 10 mg streptomycin, 25µg amphotericin B/ml, Sigma, Irvine, UK).

DMEM/F-12 (Dulbecco's modified Eagle Medium) contains; 15mM HEPES buffer, L-glutamine, pyridixine HCL, without pyridoxal HCL or phenol red. The following were added;

10 % Fetal calf serum (Hyclone, Logan, UT, USA)

5 ml antibiotics /antimycotic (Penicillin 10.000, 10 mg streptomycin, 25µg amphotericin B/ml, Sigma, Irvine, UK).

15 ml sodium bicarbonate (Sigma, St.Louis, USA).

10xAlex Jeffrey buffer (for 5ml)

1M tris (Ph8.8)	2.25ml	
1M NH ₄ SO ₄		550µl
1MmgCL ₂		225µl
100mMdNTP		100µl
20mg/mlBSA		275µl
neat (14.3m) βMercapto ethanol		23.4µl
10mM EDTA (pH=8)		2.2µl
water		1274.4µl

Appendix B

Linear discriminant analysis (LDA) is used to describe differences among groups and classify subjects into groups on the basis of battery of measurements.

LDA can be quite parsimonious when comparing different groups on different variables, as the groups may differ mainly on only 2 major dimensions i.e. the discriminant function. Each DF is a linear combination of the original variables.

There are 2 methods in interpreting the discriminant functions, either by examining the standardised coefficients (the raw coefficient for each variable is multiplied by the standard deviation for that variable), *or* by examining the discriminant function-variable correlation's, which is the correlation's between each discriminant function and each of the original variables.

To determine the directional differences among the groups, these are plotted. The horizontal direction corresponds to the first discriminant function and therefore lateral separation among the groups indicates how much they have been distinguished on this function. The vertical dimensions corresponds to the second discriminant function, where the groups are distinguished in a vertical separation, which is unrelated to their separation on the first DF. Therefore 2 groups may differ very little on the first DF, while they are separated largely on the second DF.

The DF can be rotated to help interpret them. When rotating the DF, the maximising property is lost i.e. the first rotated DF may not account for the maximum amount of between association³⁰⁸.

The mean vectors and covariance matrix are replaced by their usual estimates when the parameters are unknown. The 9 groups = k, and the responses are described by multinormal random variables with mean vectors μ_1, \dots, μ_k and a common covariance matrix Σ .

$$\hat{\mu}_j = \bar{x}_j$$

$$S = \frac{1}{N - k} \sum_{j=1}^k A_j$$

Sample vector x_j and the sums of squares and products matrix A_j for the jth group. If x is the new observation of unknown origin, the linear discriminant scores can be computed.

$$W_{ij} = x'S^{-1}(\bar{x}_i - \bar{x}_j) - 1/2(\bar{x}_i - \bar{x}_j)'S^{-1}(\bar{x}_i - \bar{x}_j)$$

Assign x to population i if $W_{ij} > 0$ for all $j \neq i$

It is noted that $W_{ij} = -W_{ji}$ and that any $k - 1$ linearly independent W_{ij} form a basis for the complete set of the statistics if $k - 1 \leq p$. If $p < k - 1$ the space of the W_{ij} will have rank p , and the classification rule can be defined in terms of p scores ³⁰⁹

Authors Publications

Based on research in this thesis

Original Publications

- **Wahab, M.**, Thompson, J., and Al-Azzawi, F. (2000) Effect of different cyclical sequential progestins on endometrial vascularity in postmenopausal women compared to the natural cycle. *Hum. Reprod.* 15, 2075-2081.
- **Wahab, M.**, Thompson, J., and Al-Azzawi, F. (2000) The effect of submucous fibroids on the dose-dependent modulation of uterine bleeding by trimegestone in postmenopausal women treated with hormone replacement therapy. *Br J Obstet Gynaecol* 107, 329-334.
- **Wahab, M.**, Thompson, J., Hamid, B., Deen, S., and Al-Azzawi, F. (1999) Endometrial histomorphometry of trimegestone-based sequential hormone replacement therapy : A weighted comparison with the endometrium of the natural cycle. *Hum. Reprod.* 14, 2609-2618.
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- **Wahab, M.**, Thompson, J., and Al-Azzawi, F. Morphometric study of endometrial blood vessels in trimegestone-based hormone replacement therapy compared to the natural cycle. *Climacteric*, 1999, 2, Supp 1, pp 297
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